Attorney Docket No.: 00.22US PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Maes, et al.

Serial No.: 09/773,351 Group Art Unit: 1617

Filed: January 31, 2001 Examiner: Cotton, Abigail Manda

For: Cholesterol Sulfate and Amino Sugar Compositions for Enhancement of Stratum Corneum Function

APPELLANT'S BRIEF PURSUANT TO 37 CFR 41.37

Assistant Commissioner of Patents and Trademarks

Attention: Board of Patent Appeals and Interferences

Washington, D.C. 20231

Dear Sir:

Appellants hereby appeal to the Board of Patent Appeals and Interferences from the final rejection of claims 1 and 3 to 20 in the present application in the decision of April 12, 2006. A notice of appeal was filed on August 10, 2006. Please see the table of contents below.

REAL PARTY IN INTEREST

The name of the real party in interest in this appeal is Color Access, Inc., the assignee of the application.

RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences relating to the instant application that would directly affect, be directly affected by, or have a bearing of any kind on the Board's decision in this appeal that are known to Appellants.

STATUS OF CLAIMS

Claims 1, and 3 to 20 remain pending and rejected in the application. All pending claims, a copy of which is attached hereto, are included in this appeal as of the submission of a Response to a non-final Office Action dated August 18, 2005 submitted on January 11, 2006.

STATUS OF AMENDMENTS

In response to the Office Actions of April 12, 2006, September 10, 2004, March 23, 2004, May 6, 2003, May 1, 2002 and in a Preliminary Amendment submitted with a Request for Continued Examination on April 12, 2002 no amendments were made to Claims 1 and 3 to 20. In Appellants' response to the Office Action of July 5, 2001, Claims 12 and 20 were amended. Claim 18 was amended in a response to the Final Office Action of December 12, 2001; and, Claims 1, 13, 16, and 19 were amended in a Preliminary Amendment submitted with a Request for Continued Examination on October 1, 2002. Claims 1, 2, 4, 7, and 10 were amended in Appellants' response to the first Office Action of June 3, 2002. Amendments were made to claims 1, 16, 18 and 19, and Claim 2 was canceled in a response to an Office Action dated November 5, 2002. Further amendments were made in a Preliminary Amendment submitted with a Request for Continued Examination on January 5, 2004 to Claims 1, 13, 16, and 19; and still further amendments were made in a Preliminary Amendment submitted with a Request for Continued Examination on May 9, 2005 to claims 1, 13, 16, and 19. Final amendments were made in Appellants' response to the Office Action of August 18, 2005 to Claims 1, 13, 16, and 19. All pending claims, a copy of which is attached hereto, are included in this appeal.

SUMMARY OF THE CLAIMED SUBJECT MATTER

The present invention relates to compositions containing an integral mixture of cholesterol sulfate and an amino sugar for the enhancement of the protective barrier of the skin and repair of the barrier if it has been damaged by chronological aging or other environmental factors. This is described in the present specification at page 1, lines 5 to 7; page 3, lines 5 to 11; and page 4, lines 11 to 24.

None of the dependent claims that are separately argued include a means plus function or a step plus function.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The outstanding issues center around one main question which is whether or not one of ordinary skill in the art would know and understand the teaching of bilayers, starting materials and components of lipid vesicles in two Ribier et al. references, (U.S. Patent Nos. 5,650,166, and 5,925,364), to be a teaching of an integral mixture of an exfoliant and cholesterol sulfate in the present invention. This main issue applies to the following four issues raised by the Examiner.

1) whether one of ordinary skill in the art would know and understand the disclosure of lipid vesicles and their bilayers, starting materials and components in Ribier et al. (U.S. Patent No. 5,650,166, hereinafter Ribier-166) when read as a whole to be a disclosure of an integral mixture of an exfoliant and cholesterol sulfate in the present invention such that it anticipates Claims 1 and 3 to 9;

2) whether Claims 1, 3, 4, 6 to 9, 11 and 18 are obvious over Ribier et. al. (U.S. Patent No. 5,925,364, hereinafter Ribier-364 in view of Sebag et al. (U.S. Patent No. 5,411,742, hereinafter Sebag) based on teachings in Ribier-364 similar to Ribier-166 of bilayers, starting materials and components of lipid vesicles such that one of ordinary skill in the art would know and understand them in combination with the teaching of cholesteryl phosphate in Sebag to be an integral mixture of cholesterol sulfate and an exfoliant of the present invention;

3) whether Claims 13 to 20 are obvious over Ribier-166 again based on teachings of bilayers, starting materials and components of lipid vesicles because one of ordinary skill in the art would know and understand them in Ribier-166 alone to be an integral mixture of cholesterol sulfate and an exfoliant of the present invention; and

4) whether Claims 10 to 12 and 20 are obvious over Ribier-166 in view of Subbiah (U.S. Patent No. 6,150,381, Subbiah) and Ichinose et al. (U.S. Patent No. 5,702,691, Ichinose) based on teachings of bilayers, starting materials and components of lipid vesicles because one of ordinary skill in the art would know and understand them in Ribier-166 in combination with Subbiah and Ichinose to be an integral mixture of cholesterol sulfate and an exfoliant of the present invention.

Each of the four sets of rejections outlined above is addressed in the Arguments section below.

ARGUMENTS

1) Disclosure of lipid vesicle bilayers, starting materials and components in Ribier et al. (U.S. Patent No. 5,650,166, hereinafter Ribier-166) would not be known and understood by one of ordinary skill in the art as an integral mixture of cholesterol sulfate and an exfoliant.

- ***Claims 1, and 3 to 9***

With respect to the anticipation rejection the Examiner finds the following in the Office Action of April 12, 2006, page 3.

> Ribier et al. discloses a moisturizing composition for the treatment of surface and deep layers of the skin clear comprising the instant ingredients such as cholesterol sulfate in the salt of alkali metal (including potassium)(see col. 3, lines 64-67), N-acetylglucosamine (see col. 5, lines 59-67), the particular sterol, cholesterol (see col. 3 line 60 and col. 6, lines 47-49), fatty acids, including linoleic acid (see col. 6, lines 44-46.) Ribier et al. further teaches the use of plant extracts (see col. 7, lines 5-8.) The compositions of Ribier et al. may be an emulsion, gel, lotion, and ointment form (see col. 7, lines 10-14.)
>
> Thus the disclosure of Ribier et al. anticipates claims 1 and 3-9. . . .

Appellants have argued that the arrangement of the components in Ribier et al. (U.S. Patent No. 5,650,166, hereinafter referred to "Ribier-166") are not as an integral mixture, and therefore, Ribier-166 fails to disclose an element of the present claims. Anticipation requires identity of invention: the claimed invention, as described in appropriately construed claims, must be the same as that of the reference, in order to anticipate. *Continental Can Co. USA, Inc. v. Monsanto Co.*, 948 F.2d 1264, 1267, 20 USPQ2d 1746, 1748 (Fed. Cir. 1991). *See also In re Spada*, 911 F.2d 705, 708, 15 USPQ2d 1655, 1657 (Fed. Cir. 1990) ("the reference must describe the applicant's claimed invention sufficiently to have placed a person of ordinary skill in the field of the invention in possession of it"). Therefore, Appellants assert that it is relevant whether features set forth in the present claims are present in Ribier-166. This is because the present invention is based on the arrangement of the ingredients as an integral mixture. The elements in Ribier-166 are not arranged as they are in the present invention and this issue has not been addressed.

The arrangement in Ribier-166 is not an "integral mixture" as one of ordinary skill in the art would understand it. Two ingredients that are separated from one another, as they are in the Ribier-166 by virtue of the vesicle formation, cannot be a mixture or be integral with one another because they are not actually combined. The Examiner has admitted in the Final Office Action of April 14, 2006 that a mixture can be interpreted by one of ordinary skill in the art as being integral with and notes that Appellants have accepted this plain meaning as well. However, Appellants assert that the present claims are limited in a way that one of ordinary skill in the art would understand that the present invention is an integral mixture separate and distinct from the separate lipid bilayers of Ribier-166. There is no integration (or mixture) where there is separation. Therefore, Ribier-166 does not anticipate the claims of the present invention because it fails to disclose an

integral mixture. In deciding the issue of anticipation, two steps must be taken: first, the elements of the claims must be identified to determine their meaning in light of the specification; and second, the corresponding elements disclosed in the allegedly anticipating reference must be identified. *Lindemann Maschinenfabrik GMBG v. Am. Hoist and Derrick Co. et al.*, 221 USPQ 481, 485; *Cf. Slimfold Mfg. Co. v. Kinkead Indus., Inc.*, 810 F.2d 1113, 1116, 1 USPQ2d 1563, 1566 (Fed. Cir. 1987). The second step has not been taken and cannot be taken because an integral mixture is not found in Ribier-166.

A. Claims Are Limited to an Integral Mixture in a Vehicle Supported by the Specification

The Examiner has noted at page 7 of the Office Action of August 18, 2005 that the present claims are not limited to "the act of mixing produce a mixture" [*sic*] with respect to the novelty rejection over Ribier-166. Further at page 7 of the August 18, 2005 Office Action, the Examiner questions what orderly manner is used to form discrete layers of a vesicle dispersed in the aqueous phase. Thus, Appellants believe that the Examiner acknowledged that Ribier-166 discloses discrete layers of a vesicle dispersed in the aqueous phase. However, according to the Examiner at page 8 of the August 18, 2005 Office Action, at the time the claims only recited a composition comprising a mixture of effective amounts of cholesterol sulfate or salts, integral with or mixed with an exfoliant in a vehicle. The Examiner again found that the features that Appellants argued and asserted were not in the rejected claims. In response, Appellants asserted that the claims previously, and as amended in the Appellants Response of January 11, 2006 to further enhance the clarity, were directed to the very claim limitation, an integral mixture in a vehicle, which is missing from Ribier-166. To advance prosecution, the claims were amended to precisely state "an integral mixture." Specifically, Claim 1 reads as follows.

> 1. A composition for topical application to the skin comprising an integral mixture of cholesterol sulfate or salts thereof present in an amount between 0.05 to about 5.00 percent, integral with an exfoliant present in an amount between 0.1 to about 10.0 percent by weight of the composition in a cosmetically or pharmaceutically acceptable vehicle.

Appellants assert that regardless of the method used to achieve their formation, the materials found in Ribier-166 discrete layers are not integral with each other, and therefore, no anticipation can be found. Based on a review of Ribier-166 as a whole, one of ordinary skill in the art would expect to make vesicles with discrete layers and not the integral mixture of components of the present invention. Because the product of the present invention is different than the alleged product taught by Ribier-166, the present claims are adequately directed to limitations that are not disclosed by Ribier-166.

The Examiner also raises at page 13, of the Final Office Action of April 12, 2006 and at page 3, of the Office Action of March 23, 2004 an issue that the integral mixture is not defined in the present specification to exclude compositions having vesicles or two or more phases. Instead, the Examiner notes that the present

specification refers to "integrated results" mentioned on page 4, lines 22 to 24, of the present specification in the sense that effects provided by each component are not canceled out. Therefore, the Examiner finds that there is no support in the specification under 35 U.S.C. §112, first paragraph, for an "integral mixture" that excludes compositions having vesicles of two or more phases. However, the Examiner does find that the term "integral mixture" would be subject to its ordinary plain meaning. Appellants respectfully traverse this rejection because the integrated result is only rendered by the integral mixture. The result is inextricably bound to the mixture because it is only by virtue of the mixture that the result is achieved. Since the plain meaning of the term integral mixture is according to the Examiner applicable, and since the mixture is like the result - integral, Appellants request that the rejection for lack of support in the specification under 35 U.S.C. 112, first paragraph be withdrawn.

B. Present invention is distinct from Ribier-166

The Examiner has argued at page 7 of the August 18, 2005 Office Action that the Ribier-166 compositions are a mixture of the ingredients in the present invention such as cholesterol sulfate in the salt of alkali metal (including potassium), N-acetylglucosamine, the particular sterol, cholesterol, and fatty acids, including linoleic acid. However, this is simply not the case. The Ribier-166 compositions are vesicles containing materials that would not be recognized by one of ordinary skill in the art as an integral mixture. In the present invention, an integral mixture of ingredients is in a cosmetic or pharmaceutical vehicle. Unlike the present invention, the Ribier-166 compositions are discrete layers in an aqueous phase. This is taught in Ribier-166 at column 1, line 65 to column 2, line 13, and column 1, lines 24 to 30, where it is explained that the Ribier-166 compositions are two types of lipid vesicles and that lipid vesicles are understood by one of ordinary skill in the art to be particles formed of a membrane of concentric lamellae where the lamellae contain bimolecular layers of amphiphilic lipids encapsulating an aqueous phase. Thus, the Ribier-166 end product of a vesicle is indeed different than the present invention. Appellants have repeatedly asserted that the method and/or steps for achieving the end result is irrelevant as long as the arrangement of the ingredients in the final product of the present invention are distinct from what is described in Ribier-166. It is not necessary to know how or why the elements in the '166 reference end up being arranged as they are. What is at issue here is that the Ribier-166 elements are not arranged as an integral mixture in a cosmetic or pharmaceutical vehicle, and therefore, Ribier-166 fails to anticipate the present invention. The elements in Ribier-166 are not arranged as they are in the claims of the present invention and this has not been addressed.

Contrary to the Examiner's assertion, the arrangement in Ribier-166 is not an "integral mixture" in a vehicle as one of ordinary skill in the art would understand it. Two ingredients that are separated from one another, as they are in the Ribier-166 by virtue of the vesicle formation, are not integral with one another. The Examiner has previously admitted in the Office Action of September 10, 2004, page 2, that a mixture can be

interpreted by one of ordinary skill in the art as being integral with. Therefore, Appellants assert that one of ordinary skill in the art would understand that the present invention is an integral mixture distinct from the separate lipid bilayers of Ribier-166. Although the steps to preparing the Ribier-166 lipid bilayers involve a stirring step, there is no disclosure that the ingredients simply stirred are added to a cosmetic or pharmaceutical vehicle. Rather, Ribier-166 discloses at column 8, lines 7 to 14, and column 8, lines 15 to 22, stirring to form vesicles that can then be added to a fatty phase. The vesicles formed in Ribier-166 are defined at column 1, lines 23 to 29 and include bimolecular layers. Additional hydration and/or dialysis treatment steps are also disclosed in Ribier-166 at column 7, lines 42 to 61. The separate layers of Ribier-166 vesicles are contrary and opposite to the integral mixture of the present invention. Therefore, Ribier-166 does not anticipate the claims of the present invention because it fails to disclose an integral mixture of cholesterol sulphate integral with an exfoliant in a vehicle, and Appellants request withdrawal of this rejection.

2) The claims of the present invention are non-obvious in view of Ribier-166

- ***Claims 13 to 20***

The Examiner notes in the Final Office Action of September 10, 2004 at page 9, that notwithstanding the fact that Ribier-166 fails to disclose the amount of each of the ingredient components of the present invention, one of ordinary skill in the art would know to use the amounts of the ingredients taught in Ribier-166. Therefore, according to the Examiner Claims 13 to 20 of the present invention are obvious. Appellants respectfully traverse this line of reasoning because Ribier-166, as discussed above with respect to the novelty rejection, fails to teach or suggest a mixture of the ingredients such that they are integral with one another.

The limitations of the claims sufficiently describe an integral mixture of components which one of ordinary skill in the art would recognize as being distinct and separate from the same components physically located in separate bilayers of a liposome (or vesicle) as they are in Ribier-166. Appellants have not found that this point has been addressed. "A proper analysis under §103 requires, *inter alia*, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out [the claimed process], those of ordinary skill would have a reasonable expectation of success." *In re Vaeck,* 20 USPQ2d 1438, 1442 (CAFC 1991); see *In re Dow Chemical Co.*, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). These two factors have not been met in the present case.

First, there is no teaching or suggestion in the prior to make an integral mixture of the pertinent components in Ribier-166. The teaching in Ribier-166 of the starting materials and components physically located in separate bilayers of a liposome is contrary and opposite to the integral mixture of the same components of the present invention. In an integral mixture, the components are not separated; but rather, are integrated. Since

Ribier-166 only teaches the components in a state of separation, namely present in separate bilayers of the liposome, the integral mixture of the present invention is not taught or suggested by Ribier-166.

The second factor of an obviousness analysis is likewise not met because Ribier-166 fails to reveal that making the composition of the present invention, namely the integral mixture of the components, would be expected by one of ordinary skill in the art to have reasonable success. This factor is linked to the first factor because as long as there is no teaching or suggestion in Ribier-166 to make the mixture of the present invention, there likewise, cannot be a reasonable expectation of success to do what is not taught or suggested. But beyond this, the teachings of Ribier-166 are aimed at treating two different layers of the skin at the same time. Thus, the components of Ribier-166 compositions start out separated in the composition and the components remain separated as they are directed to two different areas of the skin. There is never a deliberate mixing or integration of the components of Ribier-166 compositions in a vehicle of a composition. This is illustrated by the teaching at column 1, lines 11 to 14, where Ribier-166 compositions are described as comprising at least one active agent conveyed via at least two distinct types of lipid vesicles. Additional support is found at column 2, lines 19 to 21, of Ribier-166 wherein it is taught that the alleged invention involves two different agents to act in different areas of the skin. The different agents act in different areas due to the different Ribier-166 lipid vesicles containing them. The different Ribier-166 vesicles are classified based on the different types of action (see column 2, lines 34 to 41 of Ribier-166.) Every aspect of Ribier-166 compositions relates to being separate and distinct. Thus, Ribier-166 does not teach, suggest, nor motivate one of ordinary skill in the art to make the compositions of the present invention having integrally mixed components.

The contrast between the integral mixture of two ingredients in a vehicle in the present invention and the teachings of Ribier-166 is worthy of note. Ribier-166 only discloses the use of cholesterol sulfate to form lipid vesicles that can be used to encapsulate NADG. Thus, the two ingredients in Ribier-166 reference are separated by the discrete layers of the vesicle that are formed. The ingredients are separated by virtue of their being present in separate and discrete layers of the lipid vesicle. The vesicle is similar to a nut in a shell. Like NADG and cholesterol sulfate in Ribier-166, the nut and the shell are not mixed, integrally or otherwise.

Cholesterol sulfate in Ribier-166 is part of the bilayer of the liposome and resembles the shell of a nut. Inside of the shell lies the nut, which resembles NADG inside of the bilayers of the liposome. The important feature of this analogy is that the shell and the nut are not mixed just as NADG when it is encapsulated by a lipid bilayer containing cholesterol sulfate is not mixed with cholesterol sulfate. To further support the analogy Appellants submit herewith a copy of Abraham, et al., "Interaction between corneocytes and stratum corneum lipid liposomes in vitro," Biochimica et Biophysica Acta, 1021, pp. 119-25 (1990). In the article, the authors provide pictures of the liposomes which appear as bubbles in Figures 1 and 3, pp. 121 to 123. Even in looking at the liposome as a bubble or a balloon, it can be seen that cholesterol sulfate is used to make the thin layered wall of the bubble or balloon. For decorative purposes some translucent balloons are

made to hold items inside. This is similar to NADG held inside of a liposome. In each case, a balloon or a nut in a shell, the outer wall is not mixed with the content held inside. Likewise, inside of a liposome, NADG is not mixed with cholesterol sulfate as taught in Ribier-166. Therefore, the arrangement of the components taught in Ribier-166 is not the arrangement of the present invention, and thus, the present invention is not obvious in light of Ribier-166.

Cholesterol sulfate is taught at column 3, lines 57 to 67, of Ribier-166 as being an additive to the lipid membrane of the vesicle. Further, one of the actives contained within the lipid vesicle with deep down action are taught at column 5, lines 59 to 67, to include *inter alia*, NADG. Thus, there is no mixing of these ingredients as they are in the present invention by adding them in combination to a vehicle. Unlike the present invention, the Ribier-166 vesicles contain these two ingredients in separate and distinct layers and the vehicle containing them in separate layers is added to a medium as taught at column 8, lines 32 to 35. This is in contrast to adding the ingredients themselves directly to a vehicle. This is especially the case for N-acetylglucosamine (NADG) as Ribier-166 fails to teach or suggest adding the NADG directly to a vehicle because it teaches that the NADG is contained within the lipid vesicle. Therefore, even though the vesicle is added to a vehicle, because the NADG is inside the vesicle, there is no teaching in Ribier-166 where NADG, *per se*, is added directly to the vehicle. An integral mixture of NADG with cholesterol sulfate was not in the possession of one of ordinary skill in the art as Ribier-166 merely suggests that cholesterol sulfate is useful as a lipid bilayer of a vesicle holding NADG inside of it. Accordingly, the present invention is not obvious in view of Ribier-166 and Appellants request that this rejection be withdrawn.

3) The claims of the present invention are non-obvious over Ribier et. al. (U.S. Patent No. 5,925,364, hereinafter Ribier-364) in view of Sebag et al. (U.S. Patent No. 5,411,742, hereinafter Sebag)

- ***Claims 1, 3, 4, 6 to 9, 11 and 18***

With respect to the rejection of these claims, the Examiner notes at pages 5 to 6 of the April 12, 2006 Final Office Action the following.

> One having ordinary skill in the art at the time the invention was made would have been motivated to employ the particular fatty acid, linoleic acid, and cholesterol in the composition of Ribier et al, since fatty acids broadly and the particular fatty acid, linoleic acid, and known to be useful in a cosmetic or dermatological composition for treating skin based on the prior art. Moreover, cholesterol is well known to be used as a cosmetic or dermatological composition for treating skin according to Sebag. Therefore, one of ordinary skill in the art would have reasonably expected that combining the composition of Ribier et al and the composition of Sebag known to be useful for the same purpose, treating skin, in a composition to be administered would improve the therapeutic effect for treating skin, in a composition to be administered would improve the therapeutic effect for treating skin.

As noted above, Appellants have asserted that the act of mixing the starting materials and components of Ribier-166 can produce two different results, namely, one being an integral mixture and the other being a

vesicle with discrete layers. Therefore, the process is irrelevant. The same is true for Ribier-364 because its teachings are similar to that of Ribier-166. Thus, the arguments above apply to Ribier-364 as well. What is at issue in the present application is that the results of mixing are different, and that the claims are directed to features that are not present in a vesicle. Appellants respectfully traverse the line of reasoning presented by the Examiner in the Final Office Action of April 12, 2006 at page 12 with respect to Ribier-166 and Ribier-364 because they each fail to teach or suggest a unit (the composition) based on a dictionary definition of the term "integral" in the Merriam-Webster Online Dictionary (without submission by the Examiner of the definition on a Notice of References cited or a copy of the definition provided with the office action). At page 12, of the Final Office Action of April 12, 2006, the Examiner notes in a parenthetical "(formed as a unit with another part <a seat with integral headrest.)" The Examiner goes on to note that the prior art teaches and/or suggests such a composition, because cholesterol sulfate is combined with an exfoliant in a single cosmetic composition (a single unit). However, the issue is what is occurring throughout and within the unit (the composition).

The comparison that probes the issue is at the level of cholesterol sulfate and the exfoliant. In the present invention, these two components are an integral mixture in the composition, and this is in stark contrast to the Ribier et al. references where cholesterol sulfate and N-acetylglucosamine are separate and distinct and in no way are integrated throughout and within the unit (the vesicle or the composition). While it is true that the cholesterol sulfate and another component are confined within a unitary space, i.e., the vesicle or composition, this does not lead to the conclusion that the cholesterol sulfate is a unit or unified with the components in the other layers of the vesicle. This is what separates Ribier-166 and Ribier-364 from the present invention and one of ordinary skill in the art would readily know and understand this difference.

The cholesterol sulfate in one layer of the Ribier-364 vesicle is not integrally mixed with the N-acetylglucosamine of any of the other layers of the vesicle. Rather, the cholesterol sulfate layer is separated from the N-acetylglucosmine layer of the vesicle. No mixing of cholesterol sulfate in one layer of the Ribier-364 vesicle occurs with the other N-acetylglucosamine layer of the Ribier-364 vesicle. However, unlike the Ribier references, the present invention is an integral mixture of cholesterol sulfate and an exfoliant throughout and within the composition that produces the integrated result of a balanced nurturing of the skin barrier. Therefore, the integral mixture of the present invention is not taught or suggested by the Ribier references. At best, Ribier-364 or Ribier-166 could be interpreted as teaching how to bring cholesterol sulfate and N-acetylglucosamine in close proximity to one another in separate bilayers, but not as a mixture and certainly not as an integral mixture. Sebag fails to remedy the defect of Ribier-364.

A. Sebag teaching of cholesteryl phosphate in a vesicle is not an integral mixture

Both the Sebag and Ribier-364 preparations fail to produce a mixture because the ionic lipids swell under the action of mixing to form discrete layers of a lipid vesicle which separates its contents from the other ingredients in the composition, namely the outside media (e.g., the aqueous phase). The Examiner notes at page 4 of the Final Office Action of September 10, 2004 and the Final Office Action of April 12, 2006 at page 5 that Ribier-364 fails to disclose linoleic acid and cholesterol. However, these compounds are not disclosed in the present claims, and it is not clear why this is pointed out. Specifically, one of ordinary skill in the art would not substitute cholesterol with cholesterol sulfate or cholesteryl phosphate with cholesterol sulfate. In support of this assertion, Appellants submitted a declaration by Philip Wesley Wertz ("the Wertz Declaration") associated with Application No. 08/865,821 for its pertinence to the distinct nature of cholesterol and cholesterol sulfate. In paragraph 5, of the Wertz Declaration, the declarant makes the following statement.

> One of ordinary skill in the art recognizes that cholesteryl esters, cholesterol, and cholesteryl sulphate and phosphate are distinct compounds and treats each of these compounds as non-equivalent compounds due to their distinct distribution in the stratum corneum as well as their different functions in the stratum corneum.

Therefore, the Examiner's reliance on the teaching of cholesterol (specifically cholesteryl phosphate) in Sebag at column 2, line 34 is misplaced. It would not have been obvious nor does the combination of linoleic acid, cholesterol and exfoliant in the cited references make the present invention.

Appellants pointed out in their Response of June 23, 2004 at page 6, lines 5 to 7, and in their Preliminary Amendment accompanying a Request for Continued Examination submitted on May 9, 2005 that the cited references, Ribier-364 and Sebag, teach that discrete layers of a lipid vesicle are formed by mixing. If an inventor takes steps that the prior art suggests cannot be made, it is probative of non-obviousness. *Yamanouchi Pharm. Co. v. Danbury Pharmacal Inc.*, 21 F. Supp. 2d 366, 374 n. 15, 48 USPQ2d 1741, 1748 n. 15 (S.D. N.Y. 1998), *aff'd*, 231 F.3d 1339, 56 USPQ2d 1641 (Fed. Cir. 2000). Thus, based on the cited references, one of ordinary skill in the art would expect to make vesicles with discrete layers by mixing and not the mixture of the present invention. Because the result of the present invention is different than the result taught by the cited references, the claims are adequately directed to limitations that distinguish these results. It is not permissible to pick and choose only so much of any given reference as will support a given position and ignore the reference in its totality." *Lubrizol Corp. V. Exxon Corp.*, 986 F. supp. 302, 322, 7 USPQ2d 1513, 1527 (N.D. Ohio 1988). Specifically, and consistent with Ribier-166 and Ribier-364, it is indicated in Sebag at column 1, lines 38 to 54, that ionic lipids are capable of swelling in an aqueous solution to form a lamellar phase, and after stirring, to form vesicles dispersed in the aqueous solution. The Ribier-364 preparation does not produce a mixture because the ionic lipids swell under the action of mixing to form discrete layers of a

lipid vesicle which separates its contents from the other ingredients in the composition, namely the outside media (e.g., the aqueous phase). Therefore, there is no teaching or suggestion of a mixture like that of the present invention in Ribier-364 and Sebag, alone or in combination with one another.

As previously discussed in Appellants Response of June 23, 2004, neither Ribier-364 nor Sebag teaches or suggests that the act of mixing produces an integral mixture. To the contrary, the act of mixing in Ribier-364 and Sebag would be understood by one of ordinary skill in the art to cause the ionic lipid to swell and arrange itself in an orderly manner to form discrete layers of a vesicle dispersed in the aqueous phase. Thus, the ionic lipid used with other materials to make the vesicle is not integrally mixed with the content of the aqueous phase; but, rather is used to form a discrete entities present in the outside media (i.e., the aqueous phase). It is also not mixed as an end product. As previously discussed, the vesicle holds active agents within and keeps the actives separate from media outside of its walls. Creating a vesicle is akin to encapsulation where the actives inside and the materials used to encapsulate are not mixed with the outside media. Therefore, the combination of Ribier-364 and Sebag fails to teach or suggest the integral mixture of the present invention.

B. Unexpected Results

Even if the interpretation of one of ordinary skill in the art were that a lipid vesicle containing cholesterol sulfate in the membrane layer and NADG encapsulated therein was equivalent to the integral mixture of the present invention, Appellants assert that it would be rebutted by the surprising results of the present invention. The Examiner asserts in the Final Office Action of September 10, 2004, page 11, that the Example in the present specification provides no clear and convincing evidence of nonobviousness or unexpected results since it is not a direct comparison between the present invention and the cited prior art references. Appellants note that the standard is not a clear and convincing evidence of nonobviousness but, rather a clear and convincing showing of obviousness. All evidence of nonobviousness, however, must be considered. *In re Soni*, 44 USPQ2d 1684, 1687 (Fed. Cir. 1995). Unexpected results must be sufficient to overcome a clear and convincing showing of obviousness. *Richardson-Vicks Inc. v. The Upjohn Co.*, 44 USPQ2d 1181, 1188 (CAFC 1997). As previously noted, a clear and convincing showing of obviousness has not been made. However, even if such a showing were made, comparative test results are not the only evidence that can be presented to overcome a clear and convincing showing of obviousness. The unexpected result in the present invention lies in the fact that two opposite acting agents are combined and do not cancel out their activity.

As Appellants have pointed out above and in previous responses, the systems in the cited prior art and that of the present invention are not the same, and there is no reason to believe that the integral mixture of the ingredients directly in a vehicle would necessitate a comparison with a lipid vesicle as these are two

completely different systems and different arrangements of the components. To support this fact, Appellants submitted a copy of an article, Bouwstra et al., "Cholesterol sulfate and calcium affect stratum corneum lipid organization over a wide temperature range" Journal of Lipid Research, vol. 40, 2303-3212 (Dec. 1999). In the article, the authors note at page 2, lines 4 to 6, that reduced levels of cholesterol sulfate in stratum corneum contribute to desquamation, thus indicating that the presence of cholesterol sulfate would maintain the integrity of the stratum corneum and prevent desquamation. Therefore, Appellants maintain that one of ordinary skill in the art would expect a combination of cholesterol sulfate and an exfoliant to have no effect on the surface on the skin because while the exfoliant would contribute to desquamation, the presence of cholesterol sulfate would act to prevent desquamation. As previously mentioned, this benefit cannot even be addressed with the combination of Ribier-364 and Sebag because these two materials form lipid vesicles, and therefore, are not in fact mixed. Rather, they are separated such that one, the cholesterol sulfate, is part of a protective membrane that encases the other, the NADG. The whole point of the lipid vesicles/lamellar systems of the cited references is to protect and prevent the active inside from being exposed to anything else. Thus, a comparison of this kind would be futile.

While Appellants refrain from speculating on a specific method for forming separate lipid layers, Appellants note that it is indicated in Sebag at column 1, lines 38 to 54, that ionic amphiphilic lipids possess the property of forming mesomorphic phases and are capable of swelling in an aqueous solution to form a lamellar phase. Thus, after stirring in the presence of an aqueous solution, Sebag amphiphilic lipids form vesicles dispersed in the aqueous solution. Further, in the Summary of the Invention at column 2, lines 19 to 23, in Ribier-364, the alleged invention is described as being oily globules that are coated and dispersed in an aqueous phase. In other words, Ribier-364 oily globules are encapsulated, and thus, the contents of the ionic lipids in the lamellar phase are not mixed with, but rather, are separated from the aqueous phase. Further, the teaching of cholesterol sulphate in Ribier-364 is with respect to the coating for the oily globule as taught at column 2, lines 37 to 41, wherein it states that "each oily globule is individually coated with a . . . layer obtained from . . . at least one ionic amphiphilic lipid." An alkali metal salt of cholesterol sulphate is provided as an exemplary ionic amphiphilic lipid at column 3, lines 43 to 55 of Ribier-364. As the alkali metal salt of cholesterol sulphate is part of the coating of the oily globule, there is no integral mixture with an exfoliant formed by the teachings of Ribier-364. The potential exfoliants cited by the Examiner include salicylic acid, at column 15, line 6 of Ribier-364 and keratolytic agents at column 14, line 49 of Ribier-364. However, in both of these examples they are lipophilic actives contained inside the Ribier-364 oily globule. Ribier-364 lipophilic actives are, therefore, not integrally mixed with the coating on the oily globule. Thus, there is no teaching or suggestion in Ribier-364 or Sebag, alone or combined, of an integral mixture like that of the present invention and Appellants request that the Examiner's rejection of the claims based on this combination of references be withdrawn.

4) The present invention is non-obvious over Ribier-166 in view of Subbiah (U.S. Patent No. 6,150,381, Subbiah) and Ichinose et al. (U.S. Patent No. 5,702,691, Ichinose) and the term "integral mixture" is supported by the present specification.

- ***Claims 10 to 12 and 20***

These claims are rejected by the Examiner for the following reasons.

It would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the composition of Ribier et al. by the addition of sclareolide for its anti-acne properties as taught by Subbiah and by the addition of white birch extract for its anti-inflammatory properties as taught by Ichinose. The motivation for modification comes from the benefit of such properties in formulating cosmetic compositions. The missing ingredients have art-recognized suitability for the intended purpose of formulation cosmetic compositions.

Appellants respectfully traverse the line of reasoning presented by the Examiner in the Final Office Action of April 12, 2004 because Ribier-166 fails to teach or suggest a unit (the composition) as presented above with respect to Ribier-364. The line of reasoning is also traversed for the reasons presented above for the previous rejections based on Ribier-166. As noted above, the present claims are directed to an integral mixture of cholesterol sulfate in certain amounts with an exfoliant in certain amounts in a cosmetic or pharmaceutical vehicle. Subbiah teaches sclareolide-like compounds for treating disorders caused by microbials such as, for example, bacteria, and one specific disorder is acne. As disclosed in Subbiah, topical formulations containing sclareolide are generally prepared by admixing sclareolide in water and at least one organic solvent. However, this does not remedy the defect of Ribier-166 discussed above.

Since Ribier-166 teaches lipid vesicles encapsulating water soluble actives, the combination of these references at most suggests that sclareolide could be incorporated within the aqueous phase of the Ribier-166 lipid vesicles (i.e., sclareolide could be encapsulated). Because lipid vesicles are not integral mixtures, the combination of Ribier-166 and Subbiah fails to teach or suggest the present invention. Finally, Ichinose teaches flavanonol derivatives in hair nourishing and hair growth products and is cited by the Examiner for its teaching of the anti-inflammatory properties of white birch extract. However, like that of Subbiah, the teachings of Ichinose do nothing to remedy the defect of Ribier -166. In order to make out a *prima facie* case of obviousness, it must be shown that there is a suggestion or a teaching to one of ordinary skill in the art to make the combination of cited references or a reasonable expectation of success. *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991). Essentially, none of the cited references alone or in combination teach or suggest an integral mixture of cholesterol sulfate and an exfoliant in a cosmetic or pharmaceutical vehicle as an end product.

The integrated result further supports Appellants' argument above regarding the §102 and §103 rejections with respect to the Ribier references. With the Ribier references, producing an integrated result is not an issue because the cholesterol sulfate and N-acetylglucosamine remain in separate layers of a vesicle presumably in order for them to exhibit their individual activities without concern for canceling each other out with respect to their activity. However, the surprising result achieved with the present invention is the fact that the cholesterol sulfate and exfoliant do not need to be separated. In the present invention, they can be combined in an integral mixture with a vehicle and provide a benefit to the barrier of the skin surface. One of ordinary skill in the art as noted in the present specification at page 4, lines 5 to 24, would have expected the two components having opposing activities to cancel each other out in a mixture and not produce an integrated result. But, to the contrary, it has been unexpectedly found with the present invention that the two opposing components do not cancel each other out. This would never have been found based on the separated components of the Ribier references.

Subbiah teaches a method for treating microbial infections with a sclareolide-like compound, and therefore, fails to provide any teaching of a cholesterol sulfate and an exfoliant in an integral mixture. And, Ichinose teaches a flavanolol derivative for treating hair, and like the other secondary reference fails to teach or suggest a cholesterol sulfate and an exfoliant in an integral mixture like that of the present invention. The arrangement within Ribier-166 compositions is not an "integral mixture" in a vehicle as one of ordinary skill in the art would understand its plain meaning. Two ingredients that are separated from one another, as they are in Ribier-166 by virtue of the vesicle formation, are not integral with one another.

Even if the interpretation of one of ordinary skill in the art were that a lipid vesicle containing cholesterol sulfate in the membrane layer and NADG encapsulated therein was equivalent to the integral mixture of the present invention, Appellants assert that it would be rebutted by the surprising results of the present invention as discussed above. As Appellants have pointed out above, the vesicles in Ribier-166 and the integral mixtures of the present invention are not the same, and there is no reason to believe that the integral mixture of the ingredients directly in a vehicle would necessitate a comparison with a lipid vesicle as these are two completely different systems and different arrangements of the components. Therefore, Appellants maintain that one of ordinary skill in the art would not know and understand an integral mixture of cholesterol sulfate and an exfoliant based on the teachings of Ribier-166 in combination with Subbiah and Ichinose. Therefore, Appellants request that this rejection be withdrawn.

Appellants acknowledged the provisional double patent rejection made by the Examiner. However, in light of the arguments set forth above, Appellants will make a terminal disclaimer to obviate the rejection over U.S. Application No. 10/424,616, if necessary, in the event that allowable subject matter is indicated.

5) Conclusion

To recapitulate, the present invention is based on the finding that two ingredients, the cholesterol sulfate and the exfoliant, although they have opposing activities, when added as an integral mixture to a pharmaceutical or cosmetic vehicle, do not neutralize one another's activities, but rather their activity occurs in tandem, and can improve or maintain a healthy skin barrier. Of the five cited references, Ribier-166, Ribier-364, Subbiah, Sebag, and Ichinose, none of them disclose, teach or suggest an integral mixture of cholesterol sulfate and an exfoliant for an effect on the barrier repair of the skin surface. Ribier-166, Ribier-364 and Subbiah consistently teach how to make a vesicle having separate bilayers, and that a cholesterol or derivative may be used as part of the formation of the bilayer. There is no integration (or mixture) where there is separation. These three references also consistently teach that actives, such as N-acetylglucosamine (an exfoliant) can be an active inside of the bilayers. This however is not a disclosure, teaching or suggestion of an integral mixture of cholesterol sulfate and an exfoliant. Neither Sebag or Ichinose remedies the defect of the other three references in that they do not disclose, teach or suggest cholesterol sulfate in an integral mixture with an exfoliant. Due to the opposing activities of cholesterol sulfate and an exfoliant, this combination, based on the cited references is not in possession of one of ordinary skill in the art. Therefore, Appellants request that the rejections of the Examiner for the present application be withdrawn.

Respectfully submitted,

Date: October 10, 2006

Dorene M. Price, Reg. No. 43,018
Estée Lauder Companies
155 Pinelawn Road
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Melville, NY 11747
(631) 414-6087

CLAIMS APPENDIX

1. A composition for topical application to the skin comprising an integral mixture of cholesterol sulfate or salts thereof present in an amount between 0.05 to about 5.00 percent, integral with an exfoliant present in an amount between 0.1 to about 10.0 percent by weight of the composition in a cosmetically or pharmaceutically acceptable vehicle.

2 (canceled).

3. The composition of claim 1 wherein the composition contains a salt of cholesterol sulfate.

4. The composition of claim 3 wherein the salt is potassium.

5. The composition of claim 1 wherein the exfoliant is an amino sugar selected from the group consisting of N-acetyl-D-glucosamine, N-acetylgalactosamine, and a combination thereof.

6. The composition of claim 1 further comprising at least one fatty acid selected from the group consisting of butyric acid, caproic acid, octanoic acid, decanoic acid, dodecanoic acid, tetradecanoic acid, palmitic acid, stearic acid, linoleic acid and oleic acid.

7. The composition of claim 6 wherein said fatty acid is linoleic acid.

8. The composition of claim 1 further comprising cholesterol.

9. The composition of claim 1 further comprising both linoleic acid and cholesterol.

10. The composition of claim 1 further comprising sclareolide.

11. The composition of claim 1 further comprising a protease inhibitor selected from the group consisting of white birch extract, silver birch extract, *Boswellia* extract, bearberry extract, *Centella asiatica* extract, and *Pygeum africanum* extract.

12. The composition of claim 1 further comprising both sclareolide and white birch extract.

13. A cosmetic or pharmaceutical formulation for topical application of a composition to the skin, the formulation containing a mixture comprising an integral mixture of cholesterol sulfate or salts thereof in an amount from about 0.05 to about 5.00 percent, and from about 0.1 to about 10.0 percent by weight of an amino sugar selected from the group consisting of N-acetyl-D-glucosamine, N-acetylgalactosamine, and a combination thereof by weight of the composition being integral with one another in a cosmetically or pharmaceutically acceptable vehicle.

14. The formulation of claim 13 further comprising both cholesterol and a fatty acid selected from the group consisting of butyric acid, caproic acid, octanoic acid, decanoic acid, dodecanoic acid, tetradecanoic acid, palmitic acid, stearic acid, linoleic acid and oleic acid.

15. The formulation of claim 14 wherein the fatty acid is linoleic acid present in an amount less than 1 percent and the cholesterol is present in an amount less than 1 percent.

16. A method for improving or maintaining a healthy skin barrier which comprises adding an effective amount of an integral mixture to a cosmetically or pharmaceutically acceptable vehicle wherein said integral mixture comprises cholesterol sulfate or salts thereof in an amount from about 0.05 to about 5.00 percent by weight of the composition, and from about 0.1 to 10.0 percent by weight of the composition an amino sugar selected from the group consisting of N-acetyl-D-glucosamine, N-acetylgalactosamine, and a combination thereof, and applying said vehicle containing said mixture to the skin.

17. The method of claim 16 in which the mixture comprises from about 0.1 to about 2.0 percent cholesterol sulfate.

18. The method of claim 16 in which the composition comprises about 0.04 to about 1.0 percent cholesterol sulfate.

19. A method of treating or reducing damage to the skin, wherein the damage is associated with a reduction or loss of skin barrier function, which comprises adding an effective amount of an integral mixture to a cosmetically or pharmaceutically acceptable vehicle wherein said mixture comprises cholesterol sulfate or salts thereof in an amount from about 0.05 to about 5.00 percent by weight of the composition, and about 0.1 to about 10.0 percent of an amino sugar selected from the group consisting of N-acetyl-D-glucosamine, N-

acetylgalactosamine, and a combination thereof by weight of the composition, and applying said vehicle containing said mixture to the skin.

20. The method of claim 19 further comprising cholesterol sulfate or salts thereof in an amount of about 0.1 to 2.0 percent, about 0.5 to 8.0 percent of N-acetyl-D-glucosamine, cholesterol in an amount of about 0.2 to 1.0 percent, linoleic acid in an amount of about 0.2 to 1.0 percent by weight of the composition, sclareolide in an amount of about 0.001 to about 1.000 percent, and white birch extract in an amount of about 0.001 to about 1.000 percent.

EVIDENCE APPENDIX

1. The Wertz Declaration - Appellants submitted the Wertz Declaration with a Response to an Office Action dated August 18, 2005 submitted on January 11, 2006. In the Examiner's Office Action of April 12, 2006, the Examiner fails to expressly state that Appellants' Wertz Declaration is entered. It is noted by the Examiner that the Wertz Declaration is provided by Appellants (Applicants). Based on the consideration given to the Wertz Declaration in the Office Action of April 12, 2005, Appellants believe that the Wertz Declaration was entered.

2. Bouwstra et al., "Cholesterol sulfate and calcium affect stratum corneum lipid organization over a wide temperature range", Journal of Lipid Research, vol. 40, 2303-3212 (Dec. 1999) - Information Disclosure Statement (IDS) initially submitted with Appellants' Request for Continued Examination submitted on January 5, 2004, secondarily with the Request for Continued Examination submitted on May 9, 2005, and thirdly with the Response to an Office Action dated August 18, 2005 submitted on January 11, 2006. IDS initialed by the Examiner indicating consideration of citation with Office Action of March 23, 2004. Appellants believe that this document has been entered and considered.

3. Abraham et al., "Interaction between corneocytes and stratum corneum lipid liposomes in vitro," Biochimica et Biophysica Acta, 1021, pp. 119 25 (1990) - Information Disclosure Statement (IDS) initially submitted with Appellants' Request for Continued Examination submitted on January 5, 2004. IDS initialed by the Examiner indicating consideration of citation with Office Action of March 23, 2004. Appellants believe that this document has been entered and considered.

RELATED PROCEEDINGS APPENDIX

NONE

Attorney Docket No.: 96.27US PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Pelle et al.

Serial No.: 08/865,821 Group Art Unit: 1615

Filed: May 30, 1997 Examiner: Faulkner, Diedra

For: Lipid Mix for Lip Product

DECLARATION UNDER 37 C.F.R. §1.132

I, Philip Wesley Wertz, declare and say that:

1. I am a citizen of the United States and I reside at 1412 Laurel Street, Iowa City, IA 52240.

2. I am a professor at the University of Iowa, College of Dentistry located in Iowa City, IA, and I have an A.B. degree in Biochemistry from Rutgers University, New Brunswick, NJ and a Ph.D. degree in Biochemistry from the University of Wisconsin, Madison, WI. A copy of my curriculum vitae is attached hereto.

3. For over 20 years, I have taught, published and conducted research in the field of skin lipids. I have published numerous book chapters and articles related to skin lipids, including, in particular, the subject of the structure and function of lipids in the skin. I am very familiar with the scientific principles and issues related to the different structures and physiological roles of lipids in the skin and related to skin lipid research.

4. I have read U.S. Patent No. Zysman, U.S. Pat. No. 5,618,523 (hereinafter referred to as "the '523 reference'") entitled "Ceramides, Process for Their Preparation and Their Applications in the Cosmetic and Dermopharmaceutical Fields" which relates to ceramides having a particular structure and compounds containing the ceramide. One of the compositions can be an aqueous dispersion of lipid spherules composed of organized molecular layers made of the ceramide and another lipid compound. The other lipid compound can be cholesterol, or cholesteryl sulphate or phosphate. I understand that the

Examiner in the present application has asserted that "cholesteryl esters are encompassed by the generic term, cholesterol" in this reference.

5. In my opinion, the Examiner's statement is incorrect. One of ordinary skill in the art recognizes that cholesteryl esters, cholesterol, and cholesteryl sulphate and phosphate are distinct compounds and treats each of these compounds as non-equivalent compounds due to their distinct distribution in the stratum corneum as well as their different functions in the stratum corneum. The '523 reference does not make a generic disclosure of cholesterol derivatives and the '523 reference does not disclose cholesteryl esters. More specifically, as applied directly to the Examiner's statement, it is my opinion that the mere recitation of the term "cholesterol" or "cholesteryl sulphate and phosphate" does not encompass cholesteryl esters.

6. Cholesterol is a major constituent of the stratum corneum, and it is believed to play a major role in the stratum corneum by contributing to the epidermal permeability barrier function and mediating desquamation of the skin. Moreover, cholesterol is a major component of the intercellular membrane system in the cornified layer of the epidermis. This intercellular membrane system provides the permeability barrier of the skin. In contrast, the cholesterol esters found in the cornified layer are not membrane forming lipids, and at a physiologic temperature they are liquid. The cholesterol esters in the cornified layer are thought to separate into isolated liquid phase pockets, and therefore, it is thought that cholesterol esters do not function as components of the intercellular membrane system.

7. Those skilled in the art also do not consider cholesterol and cholesteryl esters to be interchangeable. More specifically, because of the different functions of the compounds in the skin, one skilled in the art, on reading about a particular use for one of these compounds, would not assume that one of the other compounds would also function for the same purpose. This is evidenced by the separate treatment, both structurally and functionally, of cholesterol and cholesteryl esters in the scientific literature, especially literature pertaining to dermatology.

8. With respect to biochemistry and biology, one of ordinary skill in the physiological and/or dermatological arts recognizes that cholesterol, and cholesteryl sulphate and

phosphate, as disclosed in the '523 reference, are each individual compounds having individual qualities and functions in a physiological and/or dermatological system. Therefore, one of ordinary skill in the art would not interpret or imply that cholesterol, as it is disclosed in the '523 reference, includes cholesteryl ester nor would one of ordinary skill in the art be led to substitute cholesterol with cholesteryl esters.

9. In my opinion, based on the compositions taught in the '523 reference, it would not have been obvious to one of ordinary skill in the art to replace the cholesterol in the compositions of the '523 reference with the cholesteryl ester in the lipid mixture of the present invention.

10. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

Date: 14 May 1999

Philip Wesley Wertz

FACULTY PROFESSIONAL BIOGRAPHY
College of Dentistry
University of Iowa

Date of preparation 12/12/98

I. Personal Data

1. Name Philip Wesley Wertz
2. Social Security # 173-42-0860
3. Birth Date 10/25/49
4. Department Oral Pathology, Radiology & Medicine
5. Present Rank Professor
6. Date appointed to present rank 7/93 Mo./Yr.

II. Higher Education, formal programs (most recent first)

Date Awarded (Mo./Yr.)	Degree	Major	Institution	Dates Attended
1976	Ph.D.	Biochemistry	University of Wisconsin	1971-1976
1971	A.B.	Biochemistry	Rutgers University	1967-1971

III. Professional Employment: Appointments (most recent first)

Mo./Yr.	Rank/Title	Institution
1993-present	Professor	University of Iowa College of Dentistry Dows Institute for Dental Research
1990-1993	Associate Professor	University of Iowa College of Dentistry and Dows Institute for Dental Research
1984-1990	Assoc. Research Sci.	University of Iowa College of Medicine Department of Dermatology University of Iowa Iowa City, Iowa
1981-1984	Assist. Research Sci.	University of Iowa College of Medicine Department of Dermatology University of Iowa Iowa City, Iowa
1979-1981	Assoc. of the Laboratory	McArdle Laboratory for Cancer Research University of Wisconsin
1976-1979	Postdoctoral Fellow	McArdle Laboratory for Cancer Research University of Wisconsin

IV. Certification and Licensure (Eligibility, stage of completion)

Board	Date

V. Honors and Awards (most recent first)

Certificate of appreciation for lecture delivered at Catholic University Graduate School, Seoul, Korea (1996)
Certificate of appreciation for presentation at the annual meeting of the Cosmetic Chemists Society (1994)
Certificate of appreciation from NIH for participation in minority apprenticeship program (1992)
A.B. with highest honors (1971)
Phi Beta Kappa (1970)

VI. Professional Appointments (consultantships, editorships, review panels, etc.; most recent first)

Year	Title
	Reviewer for: Biochimica et Biophysica Acta Canadian Journal of Microbiology Chemistry and Phisics of Lipids Journal of Investigative Dermatology Journal of Lipid Research Journal of Nutritional Biochemistry Lipids and others
1992-	Director of the Research Seminar Series
1992	External Reviewer for: Advisory Board for Research College of Dentistry The University of Illinois at Chicago
1991	Consultant for: Functions of Lipids in the Skin. Program Director: Donald T. Downing Department of Dermatology University of Iowa College of Medicine Program Project Grant Application -- not funded

1991-	Ceramide Metabolism & Transport in Keratinocyte Cultures Principal Investigator: Kathi Madison Department of Dermatology University of Iowa College of Medicine RO-1 application -- active
1991-	Effect of Nicotine on Inflammatory Mediators Principal Investigator: Georgia Johnson Department of Periodontics & Dows Institute University of Iowa College of Dentistry R-29 application -- active
1991-	Effect of Nicotine on Inflammatory Mediators Principal Investigator: Georgia Johnson Department of Periodontics & Dows Institute University of Iowa College of Dentistry Funded by Smokeless Tobacco Council -- completed
1987	External Grant Reviewer for NIH General Medicine A Study Section

VII. Committees (include chair and officer positions; most recent first)

Year	Committee	Organization
National:		
1995	Session Chairman, Presenter and Workshop Co-chairman at the Gordon Conference on the Barrier Function of the Skin	
1987	Session Co-chairman; Gordon Conference; Epithelial Differentiation and Keratinaization: The Cell Periphery.	
University:		
1996-1998	Ethics and Scientific Misconduct Commitee	
1992-1993	Biological Sciences Committee (Advisory Committee to the Vice President for Research) Pew Proposal Review Subcommittee (1992-1993)	
1997-1998	Biological Sciences Committee (Convener)	

1991-1997	Committee B (Human Subjects)
1997-	Committee A
1997-1998	Library Committee

Collegiate:

1998-	Senior Faculty Peer Review Committee
1998	Periodontics Department Self Study Review Committee
1996	Basic Science Subcommittee of the Curriculum Committee
1991-1994	Research Committee
1991-	Student Dental Research Committee
	Special Advisory Subcommittee on Design and Conduct of Retrospective and Prospective Clinical Studies (1992-)

VIII. Professional Memberships (include offices held; most recent first)

Year	Organization
1992-1993	Secretary/Treasurer of Iowa Chapter of AADR
1992-	ASBMB Congressional Correspondent
1991-	American Association for Dental Research
1989-	American Society for Biochemistry and Molecular Biology
1981-	American Oil Chemists Society
1981-	Society for Investigative Dermatology
1997-	American Association of Pharmaceutical Scientists
1998-	Controlled Release Society

IX. Areas of Research

Lipids of skin and oral epithelium -- structures, functions and metabolism
Epithelial permeability and drug delivery
Biochemistry of carcinogenesis
Essential fatty acids

X. Current Projects

Compositions and structures of lipids from oral mucosa and skin
Isolation and characterization of membrane coating granules
Lipid alteration in hyperplasia
Biochemical basis for oral mucosal drug delivery
Preparation of liposomes for drug delivery
Comparative enzymology of skin and oral epithelium.
Quantitative morphology of oral epithelia
Role of alcohol dehydrogenase in oral carcinogenesis

XI. Financial Resources (Grants and Contracts; include funded, pending, and approved but not funded applications; list most recent first in each category)

Title	Role on Project (eg: PI, Co-PI, consultant)	Dates	Amount
Federal:			
A Tissue Engineered Skin Model (RO1) Source: NIH Status: Pending Principal Investigator: Bozena Michniak Salary Support for PWW: 11% Comment: This is an RO1 Submitted from the University of South Carolina.	Contractor/Investigator	02/01/00- 01/31/03	
Local Effects of Alcohol, Aldehyde And Tobacco Carcinogens in the Pathogenesis of Oral Cancer Source: NIH/NIDR Status: Pending Principal Investigator: Christopher Squier Salary Support for PWW: 10% Comment: This is a component of a PPG.	Investigator	08/01/99- 03/31/04	
Production of Carcinogens by Oral Candida Strains Source: NIH/NIDR Status: Pending Principal Investigator: David Soll Salary Support for PWW: 5% Comment: This is a component of a PPG.	Investigator	08/01/99- 03/31/04	
Stratum Corneum Lipid Disks:A Novel Skin Model	Contractor/Collaborator	1-/01/99- 09/30/02	

Source: NIH Status: Pending Principal Investigator: Bozena B. Michniak Salary Support for PWW: 25% Comment: This is an RO1 submitted from the University of South Carolina. The major focus is a series of studies on mechanisms of penetration enhancer action using lipid disks prepared at UI. Some EPR spectra and TEM work will also be done at Iowa.			
Tissue Engineering, Biomimetics And Medical Implant Science Source: NIH Status: Active Principal Investigator: Jackie Bickenbach Salary Support for PWW: 5% Comment: Lipid composition will be one of several markers used to judge epithelial differentiation in several bioengineered systems.	Investigator	08/01/98- 07/01/03	
Biological Basis For Oral Mucosal Drug Delivery. Source: NIH/NIDR Status: Active Principal Investigator: Christopher A. Squier Salary Support for PWW: 20% Comment: The proposed experiments would identify the physiochemical parameters required for effective drug delivery through the oral mucosa and would identify permeability enhancers for use in oral mucosal drug delivery systems.	Investigator	07/01/95- 06/31/98	$474,624
Effects Of Alcohol On Oral Mucosal Permeability. Source: NIH/NIDR Status: Approved but not funded	Principal Investigator	07/01/95- 06/31/97	$70,835

Principal Investigator: Philip W. Wertz
Salary Support for PWW: 15%
Comment: The effects of alcohol on oral mucosal permeability to the tobacco carcinogen NNN would be examined. These studies could provide a mechanistic explaination for the association of alcohol and tobacco use with oral cancer.

Transmucosal Peptide Delivery. Source: NIH/NIDR Status: Approved but not funded Principal Investigator: William Abraham Salary Support for PWW: 5% Comment: This is a Small Business Technology Transfer application. If funded we would collaborate with Cygnus Therapeutics Inc of Redwood CA on the development of trans oral mucosal peptide delivery systems.	Investigator	07/01/95- 06/31/97	$100,000
Lipids And The Permeability Barrier of Oral Epithelium. Source: NIH-NIDR Status: Active Salary Support for PWW: 30% Comment: This grant would support studies on the formation, structure, permeability barriers. It would also include studies on the perturbation of barrier function by essential fatty acid deficiency.	Principal Investigator	09/01/93- 08/31/97	$403,504
Epithelial Permeability; The Effects Of Age and Alcohol. Source: NIH-NIDR Status: Completed Principal Investigator: Christopher A. Squier Salary Support for PWW: 20% Comment: This grant supports work on the structure, lipid composition and permeability of human epithelia and the effects of aging on these parameters. Also, the effects of alcohol consumption on oral epithelial properties and carcinogenesis will be studied.	Investigator	07/01/92- 06/30/95	$328,611

Structure And Function of Polar Lipids In Human Skin. — Investigator — 12/01/83-07/01/90 — $725,000
Source: NIH-NIADDK
Principal Investigator: Donald T. Downing
Salary support for PWW: 60%
Comment: PWW was involved in all phases of planning and execution of this project. This included extraction, isolation and analysis of epidermal lipids, synthesis of novel lipids, planning of metabolic experiments and formulation and testing of topical treatments.

Evaluation Of Pathogenic Factors In Acne Vulgaris. — Investigator — 04/15/81-07/01/90 — $1,341,000
Source: NIH-NIADDK
Principal Investigator: John S.Strauss
Salary support for PWW: 40%
Comment: As an Inversigator on this project, PWW was specifically responsible for isolation and analysis of polar lipids from normal skin and from patients with acne and scaling dermatoses.

College of Dentistry:
none

Other:
Mucoadhesive Liposomes — Principal Investigator — 08/30/98-04/30/99

Source: Cellegy Pharmaceuticals
Status: Active
Comment: This project is linked To a US Patent application on Mucoadhesive liposomes. ($30,000)

Lipid Dermatophysiology. — Principal Investigator — 10/30/91-12/31/94 — $105,00
Source: ConvaTec -- Bristol-Myers Squibb
Status: Completed ($56,900 in account)
Comment: This project focuses on the effects of sebaceous lipids, synthetic lipids and liposomes on

properties of the skin surface including permeability and adhesive performance. It is renewable on an annual basis at $35,000 per year.

Ceramides & Ceramidases of Skin	Principal Investigator	01/01/95-12/31/95	

Source: Gist-brocades
Status: Completed ($1,788 remaining)
Comment: This project involved several experiments in which the penetration of ceramides into stratum corneum was monitored, and the activity of ceramidase in epidermal homogenates was assayed. (Total costs - $5,000)

Lipid Analytical Service	Principal Investigator	11/15/90 -	

Source: Various Agencies
Status: Active on an intermittant basis ($40,000 currently in account)
This is a service with prospects of long term continuity and accumulation of small amounts of money in exchange for occasional advise or analytical services.

Roles of Lipids In Desquamation.	Co-Principal Investigator	07/01/86-07/01/90	$300,00

Source: The Procter & Gamble Company
Co-Principal Investigators: Donald T. Downing & Philip W. Wertz
Comment: This contract was renewed on an annual basis at $75,000 per year.

Roles Of Lipids In Cosmetic Properties of The Skin.	Investigator	4/15/81-6/30/86	$200,000

Source: Richardson Vicks Inc.
Principal Investigator: John S. Strauss
Comment: PWW was an investigator on this contract, which provided partial salary support during 1981-1983 and supplemented funds available through federal grants. The

contract was renewed on an annual basis. It started at $25,000 per year, and ended at $75,000 per year.

XII. Bibliography (attach sections in the following order, with authors in sequence; most recent first; list work that is published or "in press". Submitted work may also be listed; include journal title and date of submission; manuscripts in progress should not be included)

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Abraham W, Wertz PW, Downing DT: Preparation of liposomes and lamellar sheets from the total stratum corneum extracts. Annual meeting of the Society for Investigative Dermatology, 1988. Clin Res 36:629A, 1988.

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Burken RR, Wertz PW, Abraham W, Downing DT: The occurrence of "molecular rivets" in epidermis from different orders of vertebrates. Annual meeting of the Society for Investigative Dermatology, 1986. Clin Res 34:741A, 1986.

Wertz PW, Downing DT: Linoleic acid in epidermal acylglucosylceramide from newborn, growing and mature mice. Annual meeting of the Society for Investigative Dermatology, 1985. Clin Res 34:787A, 1986.

Madison KC, Wertz PW, Downing DT, Strauss JS: Lipid composition of cultured murine keratinocytes. Annual meeting of the Society for Investigative Dermatology, 1986. Clin Res 34:765A, 1986.

Squier CA, Cox P, Lesch CA, Wertz PW: Identification of lipids in successive epithelial strata of skin and oral mucosa. International Association for Dental Research meeting, 1985.

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Abraham W, Wertz PW, Downing DT: Linoleate-rich polar lipids of epidermis: structure determination by proton magnetic resonance. Annual meeting of the Society for Investigative Dermatology, 1985. Clin Res 33:621A, 1985.

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Wertz PW, Downing DT: The role of glycolipids in assembly of extracellular membranes in the skin. Annual meeting of the Iowa Academy of Science, 1984.

Landmann L, Wertz PW, Downing DT: Acylglucosylceramide and the epidermal permeability barrier: a liposome reconstitution study. Annual meeting of the European Anatomical Society, 1984. Acta Anatomica 120:241-242, 1984.

Cox PS, Wertz PW, Downing DT, Squier CA: Changes in lipid composition between consecutive sections through pig epidermis. Annual meeting of the British Society for Investigative Dermatology, 1984.

Downing DT, Stewart ME, Wertz PW, Colton SW 6th: Analysis of waxes from vertebrate skin. Annual meeting of the American Oil Chemists Society, 1984. J Amer Oil Chem Soc 61:666, 1984.

Clancey CJ, Van Orden DE, Zlatnik FJ, Wertz PW, Dowining DT: Quantitation of amniotic fluid phospholipids: A novel determination of disaturated lecithins (SPC). Annual meeting of the Society for Gynecologic Investigation, 1983.

Wertz PW, Downing DT: Epidermal sphingolipids: strucutres and possible functions in the epidermal water barrier. Annual meeting of the Society for Investigative Dermatology, 1983. Clin Res 31:608A, 1983.

Wertz PW, Downing DT: Studies on the role of glycolipids in the assembly and maintenance of the extracellular membranes in the skin of terrestrial vertebrates. Twelfth International Congress of Biochemistry, 1982.

Wertz PW, Downing DT: The structures and physiological role of lipids in the epidermal barrier to percutaneous water loss. Annual scientific meeting of the Society of Cosmetic Chemists, 1982.

Wertz PW, Mueller GC: A role for protein alkylation in phorbol ester action. Annual meeting of the American Association for Cancer Research, 1981. Proc Amer Assoc Cancer Res 22:137, 1981.

Wertz PW, Mueller GC: Activation of CTP:phosphorylcholine cytidyl transferase by 12-0-tetradecanoylphorbol-13-acetate (TPA) in bovine lymphocytes. Annual meeting of the American Association for Cancer Research, 1980. Proc Amer Assoc Cancer Res 21:511, 1980.

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Mueller GC, Kensler TW, Wertz PW, Kwong CH: Retinoic acid inhibition of phorbol ester mediated comitogenesis, phospholipid metabolism and capping in bovine lymphocytes. Cold Spring Harbor Symposium on Phorbol Esters, 1978.

Wertz PW, Mueller GC: 12-0-Tetradecanoylphorbol-13-acetate stimulates phosphatidyl choline metabolism in bovine lymphocytes. Annual meeting of the American Society of Biological Chemists, 1978. Fed Proc 37:1494, 1978.

Wertz PW: A computer assisted kinetic analysis of the tautomerization and complex mutarotation of D-galactose. Doctoral dissertation, University of Wisconsin, Madison (1976). Dissertation Abstracts International 37:4450-B, 1977.

Anderson L, Wertz PW: Correlation between the complex mutarotation of an aldose and the kinetics of its tautomerization. Centennial meeting of the American Chemical Society, 1976.

5. Invited External Presentations and Lectures

Presented an invited lecture titled "Regional variation in lipids and barrier function in keratinizing epithelia" at the conference on Perspectives on Percutaneous Penetration in Leiden, September, 1998.

Seminars at the University of Modena, University of Leiden, University of South Carolina, Medical University of South Carolina, University of California at San Francisco and University of Iowa College of Dentistry and College of Medicine, 1998.

Invited speaker at the Gordon Conference on the Barrier Function of Mammalian Skin, New Hampshire, 1997.

Invited speaker at the World Congress of Dermatology, Sydney, Australia, 1997.

Presented an invited lecture titled "Skin lipids in health and disease" at Catholic University, Seoul, Korea, August, 1996

Presented an invited lecture titled "Effects of lipids on the cosmetic properties of the skin" at Pacific Research and Development Center, Seoul, Korea, August, 1996

Presented an invited lecture titled "Lipids and the epidermal permeability barrier" at the annual meeting of the Korean Society for Investigation of the Barrier Function of the Stratum Corneum, Seoul, Korea, August, 1996

Presented an invited lecture titled "Chemistry and composition of lipids from the skin surface, stratum corneum and hair" at Amway Research Center, Grand Rapids, MI, October 1995

Presented an invited lecture titled "Lipids and the epidermal permeability barrier" at ALZA Corporatiuon, Palo Alto, CA, September 1995

Invited speaker at the Gordon Conference on Barrier Function of Mammalian Skin. Also co-directed an afternoon workshop on the ROLES OF LIPIDS IN BARRIER FUNCTION.

Presented an invited lecture titled "Lipids and the epidermal permeability barrier" at the annual scientific meeting of the Society of Cosmetic Chemists, New York, 8-9 December, 1994.

Presented an invited lecture titled "Antimicrobial lipids from the skin surface" at ConvaTec, a subsidiary of Bristol-Myers Squibb, Princeton, NJ, December 1994.

Served as co-chairman at the Dental Student Research Conference, 1990, 1992 & 1994.

Presented an invited lecture titled "Epidermal lipids -- opportunities for transdermal drug delivery and improved skin adhesives" at ConvaTec, a subsidiary of Bristol-Myers Squibb, Princeton, NJ, October 1993.

Presented an invited lecture titled "Skin lipids in health and disease" at The 6th International Colloquium on Phospholipids, Hamburg, Germany, 24-27 October, 1993.

Presented an invited lecture titled "The epidermal permeability barrier -- biochemical and ultrastructural considerations" Second Annual New Technologies Workshop: Skin Toxicology And Pharmacology -- In Vitro And Clinical Techniques, Microbiological Associates, Inc., Bethesda, MD, 9-10 September 1993.

Presented an invited lecture titled "Roles of lipids in formation and function of the epidermal permeability barrier and the influence of skin surface lipids on adherence of Candida albicans" at the ConvaTec Skin Care Symposium, Princeton, NJ, 14 May 1993

Presented an invited lecture titled "Composition, structure and organization of lipids in stratum corneum" at The Winter Workshop (The Physical Organization of Stratum Corneum Lipid) held at the University of British Columbia, Vancouver, Canada, February 28 - March 2, 1993

Served as a judge of the Max Smith Award competition at the 1992 annual meeting of the Iowa Chapter of the American Association for Dental Research

Speaker at conference on the effects of aging on oral mucosa and skin held in Iowa City, May 1992.

Presented an invited lecture titled "Lipid nature of the epidermal permeability barrier" at ConvaTec, a subsidiary of Bristol-Myers Squibb, Princeton, NJ, 1991.

Speaker and session chairman at International Conference on Liposome Dermatics in Bad Griesbach, Germany, October 1991.

Presented invited lecture titled "Essential fatty acids and epidermal integrity" at the 36th Annual Symposium on the Biology of the Skin sponsored by the Cutaneous Biology Foundation, Inc., Salishan Lodge, Oregon, 1986.

Presentations on epidermal lipids at Vicks Research Center, Shelton, CT, in 1982, 1983 and 1984.

Presented and invited lecture title "Strucutre and function of the polar lipids of epidermis" at the Gordon Conference on Epithelial Differentiation and Keratinization, 1983.

Presented invited lecture titled "The structure and physiological role of lipids in the epidermal barrier to percutaneous water loss" at the annual meeting of the Society of Cosmetic Chemists, New York. 1982.

6. Other: Exhibits, films, tapes, special presentations

XIII. Student Mentoring:

1. Graduate Theses Directed:

Degree	Year	Candidate	Title
M.S. (Co-Directed with Pete Fotos)	1993	Sandra Law	Adherence of Candida albicans to porcine stratum corneum in vitro.
M.S.	1992	Albert Manganaro	Effects of permeabilizers on penetration of propranolol through buccal epithelium in vitro.
M.S.	1997	Patricio Jarpa	Variations of pH, nicotine levels and mutagenicity of smokeless tobacco products from U.S., India and Venezuela

I have served or am serving on the thesis committees of:

Deborah Vezeau
David Rolf
Thomas Poore
Riaz Ali
Bruce Ringdahl
Hudda Hamaddi
Mike Huberman
Joseli Alves
Deena Kuempel
Earnest Lam
Kaaren Vargas
Heather Reid
Xiobing Du

2. Other Graduate Committee Service:

Degree	Year	Candidate	Title

3. Predoctoral Student Research Mentorship:

Student	Year	Awards Received by Student
Bennedicte van den Bergh (Dutch Ph.D. student)	1998	
Jennifer Garza	1997-	Dows Award NIDR Award
Matt Howard	1998 -	NIDR Award
Manal Ahmad	1997 -	DRA
Rebecca Guo	1998 -	Dows Award
Shannon Li	1998 -	DRA
Kevin Ortale	1994-1997	Max Smith Award NIDR Awards
Janet Hoogstraate (Dutch PhD student)	1994	
Sara Whittle	1995-1998	NIDR Awards

Trace Lund (David Drake Co-Mentor)	1994-1997	DRA
Matt Grau (Kevin Donly Co-Mentor)	1996	Director's Award
Doug Squier (predental 1995, dental student 1996-)	1995-	Dows Award DRA
Delon Gilbert (Predental, dental student 1997-)	1996-1997	Howard Hughs Fellowship Dows Award AADS Gies Award
Keith Slaymaker (Predental)	1996-1998	
Matt Abraham (Predental)	1996	
P. Bayati	1993-1995	Director's Award Dows Award
Ajanet Mclain (SSTP)	1994	
G Gell	1993-1995	Dows Award
Candace Wright (SSTP)	1993	
Aima Ahonkai (SSTP)	1993	
Felicia Chang	1991-1994	Dows Awards
Niki Holbrook (SSTP)	1992	
Harry Fang (local high school student; Science Research 91:86)	1992	
Nate Stevens (Predental)	1991	
William B Shade II (SSTP, CA Squier Co-Mentor)	1991	

Karen N. Ekpenyong 1991
(SSTP, CA Squier Co-Mentor)

Sofia F. Betancourt 1991
(SSTP, David Drake Co-Mentor)

Jennifer Haake 1991
(local high school student; Science Research 91:86)

Jeffrey T Bortz 1990
(Medical Student)

Christopher L Hedberg 1989
(Medical Student)

Kathy M Perisho 1988
(Medical Student)

Mark A Wix 1987
(Medical Student)

Jeffrey C Melton 1987
(Medical Student)

Russel R Burken 1983-1984
(Medical Student)

Susan Rubes 1986
(Medical Student)

Paul M Stover 1986
(Medical Student)

Saundrett Gibbs 1985
(Medical Student)

Dan Johnson 1985
(Medical Student)

Marianne C Miethke 1985
(Medical Student)

Sherri Long 1985

Best Basic Science Presentation
Best Clinical Presentation
Best Presentation in Dermatology
All at the 1986 Medical Student
Research Symposium at Galviston

Richard T Schoephoerster (Undergraduate)	1985
Mark L Frost (Medical Student)	1984
Craig Birkby (Medical Student)	1982

XIV. Continuing Education (sponsored by the University; presented during the past five years most recent first)

Presentations qualifying for continuing education credit at:
Dermatology meetings & symposia 20
Clinical research meeting 1
Seminars 2

XVI. Teaching Activities (Include the past five years; most recent first; provide narrative describing responsibility in each course)

1. Classroom, Seminar, or Teaching Laboratory

Year	Course Title and No.	No. registered	length of course
1996-	Systemic Disease Manifestations 86:155 facilitator for problem based learning	75	16 wks
1993-	Pathophysiology of Skin & Mucosa 112:251	9	16 wks
1992-95	Dental Biochemistry 99:161	70	16 wks
1991-	Dental Science Research Methodology 151:210 Course Director	31	8 wks
1991-	Research Seminar Series 151:200 Director	15	16 wks 100%
1991-	Pathophysiology of Salivary	12	16 wks 10%

	Glands 112:254		
1990-91	Dental Science Research Methodology 112:210	25	8 wks 10%
1982-90	Basic Science for Dermatology College of Medicine	12	16 wks 25%
1982-90	Research in Dermatology 62:4	6	16 wks 35%

2. Clinical Teaching (undergraduate, graduate and direct patient care)

Year	Where Occurred	weeks/year	hrs/wk

Students supervised:
2 local high school students
7 SSTP students
6 University of Iowa undergraduates
14 medical students
14 dental students
3 College of Dentistry MS students
1 high school science teacher
2 postdoctoral associates
2 Dutch PhD students
see pages 25-32 for details.

Attorney Docket No.: 00.22US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor: MAES, et al.

Serial No.: 09/773,351 **Group Art Unit:** 1617

Filed: January 31, 2001 **Examiner:** JIANG, Shaojia A.

For: Cholesterol Sulfate and Amino Sugar Compositions for Enhancement of Stratum Corneum Function

Transmittal of Second Supplemental Information Disclosure Statement under 37 C.F.R. 1.97(c)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with 37 C.F.R. 1.56, 1.97 and 1.98, Applicant submits herewith references which he believes may be material to the examination of this application and with respect to which there may be a duty to disclose in accordance with 37 C.F.R. 1.56.

While the references may be "material" under 37 C.F.R. 1.56, it is not intended to constitute an admission that the references are "prior art" unless specifically designated as such.

The filing of this Information Disclosure Statement (IDS) shall not be construed as a representation that no other material references exist or that a search has been conducted.

These references are listed on the enclosed Form PTO/SB/08A (substitute for Form 1449/PTO) which is in accordance with the requirements of M.P.E.P. 609. Copies of required English-language abstracts and English-language publications, if any, are enclosed for each foreign language reference to satisfy the requirement regarding a concise explanation of foreign language references.

1) JP11-005742, 01/12/1999, Shiseido Co. Ltd. (Abstract)
2) US 6,589,945 B1, 07/08/2003, Wachter, et al. (Counterpart of WO 98/17244)
3) US 5,989,568, 11/23/1999, Breton, et al. (Counterpart of JP08-231342 A)
4) WO88/01274 , 02/25/1988, Broadbent (Counterpart of JP Patent Publication JP02-503554 A)
5) JP2001-002551, 01/09/2001, Kanebo Ltd. (Abstract)
6) JP59-013708, 01/24/1984, Shiseido Co. Ltd. (Abstract)

It is respectfully requested that these references be considered by the Patent and Trademark Office in its examination of the above-identified application and be made of record therein. The Examiner is also invited to contact the undersigned if there are any questions concerning this paper or the attached references.

This IDS is being submitted after the first Office Action on the merits of this application, but prior to Final Rejection under 37 C.F.R. 1.113, or a Notice of Allowance under 37 C.F.R. 1.311. A fee of $180.00 under 37 C.F.R. 1.17(p) is paid with this submission. If any other fee should be required for the submission of this IDS, please charge Deposit Account No. 05-1320; a duplicate copy of this sheet is enclosed.

Respectfully submitted,

Date: January 10, 2006

Dorene M. Price (Reg. No. 43,108)
Estee Lauder Companies
155 Pinelawn Road
Suite 345 South
Melville, NY 11747

Attorney Docket No.: 00.22US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor: MAES, et al.

Serial No.: 09/773,351 **Group Art Unit:** 1617

Filed: January 31, 2001 **Examiner:** JIANG, Shaojia A.

For: Cholesterol Sulfate and Amino Sugar Compositions for Enhancement of Stratum Corneum Function

Transmittal of Second Supplemental Information Disclosure Statement under 37 C.F.R. 1.97(c)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with 37 C.F.R. 1.56, 1.97 and 1.98, Applicant submits herewith references which he believes may be material to the examination of this application and with respect to which there may be a duty to disclose in accordance with 37 C.F.R. 1.56.

While the references may be "material" under 37 C.F.R. 1.56, it is not intended to constitute an admission that the references are "prior art" unless specifically designated as such.

The filing of this Information Disclosure Statement (IDS) shall not be construed as a representation that no other material references exist or that a search has been conducted.

These references are listed on the enclosed Form PTO/SB/08A (substitute for Form 1449/PTO) which is in accordance with the requirements of M.P.E.P. 609. Copies of required English-language abstracts and English-language publications, if any, are enclosed for each foreign language reference to satisfy the requirement regarding a concise explanation of foreign language references.

1) JP11-005742, 01/12/1999, Shiseido Co. Ltd. (Abstract)
2) US 6,589,945 B1, 07/08/2003, Wachter, et al. (Counterpart of WO 98/17244)
3) US 5,989,568, 11/23/1999, Breton, et al. (Counterpart of JP08-231342 A)
4) WO88/01274 , 02/25/1988, Broadbent (Counterpart of JP Patent Publication JP02-503554 A)
5) JP2001-002551, 01/09/2001, Kanebo Ltd. (Abstract)
6) JP59-013708, 01/24/1984, Shiseido Co. Ltd. (Abstract)

It is respectfully requested that these references be considered by the Patent and Trademark Office in its examination of the above-identified application and be made of record therein. The Examiner is also invited to contact the undersigned if there are any questions concerning this paper or the attached references.

This IDS is being submitted after the first Office Action on the merits of this application, but prior to Final Rejection under 37 C.F.R. 1.113, or a Notice of Allowance under 37 C.F.R. 1.311. A fee of $180.00 under 37 C.F.R. 1.17(p) is paid with this submission. If any other fee should be required for the submission of this IDS, please charge Deposit Account No. 05-1320; a duplicate copy of this sheet is enclosed.

Respectfully submitted,

Date: January 10, 2006

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PTO/SB/08A (07-05)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	09/773,351
Filing Date	January 31, 2001
First Named Inventor	MAES, et al.
Art Unit	1617
Examiner Name	JIANG, Shaojia A.
Attorney Docket Number	00.22US

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-			
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
		JP11-005742 (Abstract)	01-12-1999	Shiseido Co. Ltd.		
		US 6,589,945 B1 --	07-08-2003	Wachter, et al.		
		(Cntrprt WO98/17244)				
		US 5,989,568 --	11-23-1999	Breton, et al.		
		(Cntrprt JP08-231342 A)				

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Sheet 2 of 2

Complete if Known	
Application Number	09/773,351
Filing Date	January 31, 2001
First Named Inventor	MAES, et al.
Art Unit	1617
Examiner Name	JIANG, Shaojia A.
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U. S. PATENT DOCUMENTS

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		WO88/01274 --	02-25-1988	Broadbent		
		(Cntrprt JP02-503554 A)				
		JP2001-002551 (Abstract)	01-09-2001	Kanebo Ltd.		
		JP59-013708 (Abstract)	01-24-1984	Shiseido Co. Ltd.		

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This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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PA 02-504

English abstract of reference A1

PATENT ABSTRACTS OF JAPAN

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(43)Date of publication of application : 12.01.1999

(51)Int.Cl. A61K 31/575
A61K 7/00
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(21)Application number : 09-218288

(22)Date of filing : 30.07.1997

(71)Applicant : SHISEIDO CO LTD

(72)Inventor : SATO JUNKO
DENDA MITSUHIRO
KOYAMA JUNICHI

(30)Priority

Priority number : 09117508 Priority date : 21.04.1997 Priority country : JP

(54) EXTERNAL PREPARATION CONTAINING CHOLESTEROL SULFATE

(57)Abstract:

PROBLEM TO BE SOLVED: To obtain the subject dermal external preparation for controlling decomposition of desmosome in the skin by including a cholesterol sulfate.

SOLUTION: This external composition is obtained by including a cholesterol sulfate (cholesterol 3-sulfate ester) derived from a living body or partially or totally synthesized at approximately 0.005 to 20 wt.%, based on the whole composition, preferably 0.5 to 5 wt.%, for lotion, cream or the like, and further including a diluent or aid (alcohol, water, chelating agent, urea, surfactant or the like) which is commonly used for cosmetics and external medicines.

LEGAL STATUS

[Date of request for examination] 29.05.2002

[Date of sending the examiner's decision of rejection]

[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

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[Date of registration]

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[Date of extinction of right]

CORRECTED VERSION*

Counterpart of reference A4

PA02-504

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :		(11) International Publication Number: WO 88/ 01274
C07J 31/00, A61K 31/575	A1	(43) International Publication Date: 25 February 1988 (25.02.88)

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(22) International Filing Date: 21 August 1987 (21.08.87)

(31) Priority Application Number: PH 7614

(32) Priority Date: 21 August 1986 (21.08.86)

(33) Priority Country: AU

(71) Applicant *(for all designated States except US)*: BROADBENT, James, Meredyth [AU/AU]; Suite 2, 227 Burwood Road, Hawthorn, VIC 3122 (AU).

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(72) Inventors; and

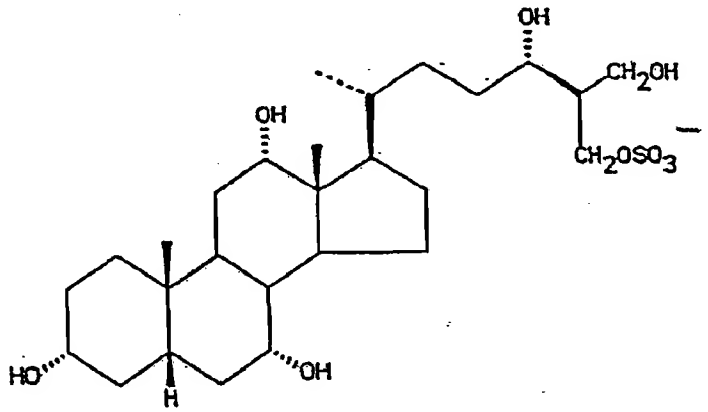
(75) Inventors/Applicants *(for US only)*: KOSUGE, Takuo [JP/JP]; 3-4-18, Kamiashiarai, Shizuoka (JP). TSUJI, Kuniro [JP/JP]; 2-11-17, Kamiashiarai, Shizuoka (JP). ISHIDA, Hitoshi [JP/JP]; 200-16, Sena, Shizuoka (JP).

(74) Agents: SLATTERY, John, Michael et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: ACTIVE PRINCIPLE ISOLATED FROM SHARK TISSUES



(I)

A+

(57) Abstract

A compound of general formula (I), in substantially pure form, wherein A is a cation. A method for preparation is also disclosed, together with compositions and methods of use thereof.

*(Referred to in PCT Gazette No.05/1990, Section II)

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C07J 31/00, A61K 31/575 — A1

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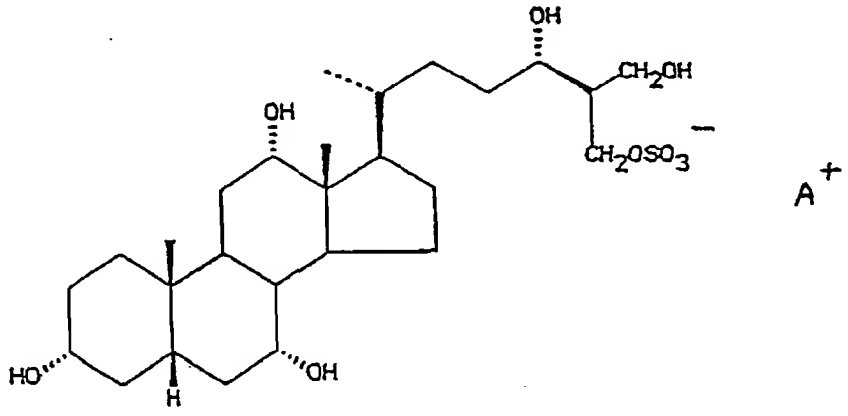
Published
With international search report.

(54) Title: ACTIVE PRINCIPLE ISOLATED FROM SHARK TISSUES



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(57) Abstract

A compound of general formula (I), in substantially pure form, wherein A is a cation. A method for preparation is also disclosed, together with compositions and methods of use thereof.

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"ACTIVE PRINCIPLE ISOLATED FROM SHARK TISSUES"

This invention relates to the identification, isolation and preparation of an active principle by extraction from natural tissues, and in particular it relates to the identification, isolation and preparation of such an active principle by extraction from particular tissues of sharks.

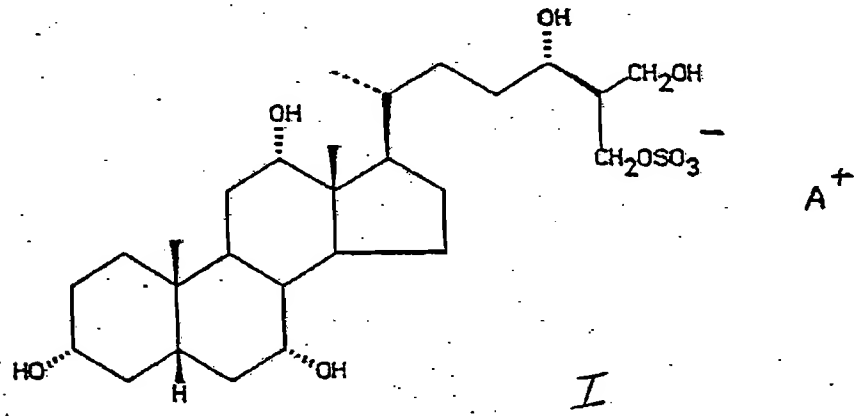
In Japan, a preparation known as "deep-sea shark liver oil" has been used as a folk remedy for a long time. It is an oil prepared from shark's liver and is normally capsulated in soft capsules. The liver oil is said to be effective in treatment of many kinds of diseases, especially those which are related to the liver, such as hepatitis, nephritis, diabetes, etc. As well, when used externally, it is widely recognised that the liver oil is effective in treatment of scalds, burns or other types of skin trouble, and also is ideal as an ingredient for cosmetics.

The present inventors have been studying this material for many years, and recently have discovered the unexpected fact that an active substance exists in the aqueous component of shark's liver rather than the oil soluble component. This fact was recognised from a comparison of the practical use of the liver oil and a powder produced from the

aqueous component of the liver by evaporation of the water. In comparative tests of a dosage of 900mg of the liver oil per day and 60mg of the powder per day, the latter gave a better clinical result than the former. Furthermore, where the liver oil was thoroughly washed with water, the resulting oil showed almost no effect. These facts indicate that the active substance of deep-sea shark liver is not oil-soluble as previously believed, but is water-soluble.

According to the present invention, there is provided an active principle which is isolated from an aqueous extract of the liver and/or gallbladder of a shark.

In a first aspect of the invention, there is provided a compound of the general formula I, in substantially pure form,



I

wherein A is a cation, such as a sodium, potassium, calcium or ammonium ion, or an organic amine.

In other aspects, this invention provides a method for the preparation of a compound of general formula I in substantially pure form, together with

compositions for pharmaceutical, dietary or cosmetic purposes which comprise such a compound.

By using activity assays which are described in detail below, it has been shown that the active principle is water-soluble and does not exist in the oil-soluble component of shark's liver. These assays have been used in a series of tests to ascertain whether the active principle exists only in the liver. All parts of the shark's body, such as the bones, meat, gallbladder, ovary, alimentary canal, etc., have been investigated, and it has been found that the gallbladder showed the same activities as liver in the assays. This result indicates that the active principle exists only in liver and gallbladder.

In general terms, the two bioassays referred to herein and used to identify sources of the active principle and to assess the degree of purity of an extract, are designed to identify characteristic pharmacological activities of the substance. In particular, the bioassays, designated as (A) and (B) are based on the following activities:

(A) The active principle prevents liver trouble in mice caused by carbon tetrachloride.

(B) The active principle increases the respiration rate in mice when a toxic substances such as nicotine is administered.

The present invention also provides a method for preparing an active principle as described above, which comprises the steps of preparing an aqueous extract of the liver and/or gallbladder of a shark, and isolating the active principle from the aqueous extract.

The following description sets out general procedures for isolation of the active principle from

the aqueous extract of the liver and/or gallbladder of a shark, involving the steps of extraction with polar organic solvents, adsorption on suitable adsorbents and/or chromatography techniques.

In order to determine whether the active principle is soluble in polar organic solvents, such as methanol, ethanol, acetone, etc., the powder obtained by freeze-drying of shark's bile was extracted with polar organic solvent, then the (A) and (B) assays were applied to both the soluble part and the insoluble part. Activity was seen only in the assays on the soluble portion, thus establishing that active principle is soluble in polar organic solvents.

In testing to determine whether the active principle can be isolated utilising adsorbents, many adsorbents were examined and it was found that the active principle can be adsorbed by ion exchange resins of basic anion exchange type, or by synthetic adsorbents such as XAD, HP-20, Sep-pak c18, etc., or charcoal. This absorption test was performed by extracting shark's liver and/or gallbladder with water. Each adsorbent under test was added to the extract and left to stand overnight. The mixture was then filtered and each filtrate tested for activity by the (A) and (B) assays. The results indicate that the active substance is adsorbed by those adsorbents mentioned above. The active principle may be recovered from the adsorbent resins by extraction with acid, alkali or salts, and from the synthetic adsorbents and charcoal by extraction with polar organic solvents.

Further purification of the active principle is achieved by chromatography, for example in a silica column, Sephadex LH-20 column, or by preparative TLC

(thin layer chromatography) or HPLC (high performance liquid chromatography), etc. Each method gave satisfactory results, but HPLC gave the best purification. The active principle as isolated by HPLC was quite pure because it gave very sharp single peak and also gave a single spot of approximate representative Rf value of 0.36 on TLC. The active principle in its purified form is a white powder of melting point of 140°C.

Testing of the purified active principle by vanillin sulfuric acid gave a purple colour, indicating that it contains bile acid or bile alcohol in its structure. It has already been found that the bile of sharks contains a bile alcohol named scymnol. After partial acetylation of the active principle with acetic anhydride, followed by treatment of the crude product with dry dioxan-trichloroacetic acid for several days, scymnol was identified from the reaction mixture. The result indicated that the active principle is a scymnol derivative. It was the first isolation of the pure scymnol derivative contained in bile of shark, as the active principle.

A preferred procedure for isolation of the active principle from the lyophilized bile of Rhizoprinodon acutus (obtained by homogenization and freeze-drying of gall-bladders), is set out in the following chart:

Lyophilized bile of Rhizoprionodon acutus
extracted with 1. n-Hexane (100mlx3)
2. MeOH (100mlx3)

Fraction I(MeOH-extract)
1. dissolved in H_2O
2. Amberlite XAD-2 c.c., eluting with
i. H_2O (400ml)
ii. MeOH (400ml)

Fraction II(MeOH-eluate)
1. dissolved in $CHCl_3$-MeOH(1:1)
2. Sephadex LH-20 c.c. eluted with
i. $CHCl_3$-MeOH(1:1) (300ml)
ii. MeOH (500ml)

Fraction III
HPLC: YMC-Pack A-324 (ODS)

Colorless powder (compound I)

As set out above, in this procedure the lyoophilized material is deflated with n-hexane, and then extracted with methanol. The concentrate thus obtained is applied to an Amberlite XAD-2 column in batches, using H_2O, and ethanol as eluents. As the ethanol eluate contains the active principle (as determined by color reagent), this fraction is successively subjected to gel filtration on Sephadex LH-20 with chloroform-methanol and methanol. The active principle is so effectively contained in the methanol eluate that its final purification is achieved by successive application of HPLC with a reverse phase column.

It has been suggested that scymnol might be in the form of a sulphate ester, but no positive information has been published about the position of attachment of the sulphate ester, because scymnol has six hydroxyl groups where the sulphate ester group might be attached. The present scymnol derivative has never been isolated as a pure substance. The active powder as purified by HPLC was subjected to elementary analyses. Results were anal: calcd for $C_{27}H_{51}O_9NS$, C;57.34, H;9.02, N;2.47, S;5.66. Found C;57.23, H;8.92, N;2.45, S;5.30. These results suggested that the active compound has ammonium sulphate ester in the structure. Nuclear magnetic resonance spectroscopy of the active powder showed the following properties.

^{1}H-NMR(in d_4-MeOH) δ(ppm):
4.22(dd, 1H, J=4.5 and 10.0Hz),
4.11(dd, 1H, J=10.0 and 16.7Hz),
4.00(bs, 1H), 3.80(d, 1H, J=1.2Hz),
3.60-3.80(m, 4H), 3.30-3.45(m, 1H), 0.72(s, 3H).

^{13}C-NMR(in d_4-MeOH) δ(ppm): 74.1(d), 72.9(d), 71.3(d), 69.1(d), 66.7(t), 61.2(t), 48.4(d), 47.8(d), 47.5(s), 43.1(d), 43.0(d), 41.0(d), 40.4(t), 37.0(d), 36.5(t), 35.9(s), 35.8(t), 33.3(t), 32.1(t), 31.2(t), 29.6(t), 28.8(t), 27.9(d), 24.3(t), 23.2(q), 18.1(q), 13.1(q).

^{13}C-NMR spectrum shows that the active compound has 27 carbon atoms made up of three methyl, 11 methylene, 11 methine and two tertiary carbons. The signals at low field (0.72-2.35) in ^{1}H-NMR spectrum suggest that it seems to be a coprostane derivative. At the higher field in ^{13}C-NMR spectrum, signals at 74.1(d), 72.9(d), 71.3(d) and 69.1(d) are

assignable to the methine carbon with hydroxyl group. And the two signals at 66.7(t) and 61.2(t) are ascribable to the O-substituted methylene carbon. 2D0COSY0NMR spectra and C-H-shift-COSY relationship indicate that these two carbons attach to a methine carbon and one of them with low chemical shift (66.7) has two unequivalent protons at 4.22(dd) and 4.14(dd)ppm in the ^{1}H-NMR spectrum, which indicates that the active compound has the partial moiety of $HOCH_2$-CH-CH_2OR in the molecule. From the results of elementary analyses, R is $-SO_3NH_4$.

From these NMR spectra and elementary analyses, the powder is characterised as 3α, 7α, 12α, 24ξ, 26-pentahydroxycoprostane-27-ammonium sulphate ester. The ammonium ion in the structure possibly came from the phosphate ammonium buffer used as mobile phase in HPLC, by replacement of a sodium ion. To verify this point, an active powder purified by XAD-2 and then by column chromatography on Sepadex LH-20 was subjected to atomic absorption spectrophotometry for sodium and to elementary analysis for nitrogen. The results were, calcd. for $C_{27}H_{47}O_9SNa$, Na;4.03, N;0.00, found Na;3.57, N;0.02. The stereochemistry of the C-24 position in the structure was determined as 24R by X-ray crystallographic analysis of scymnol and the specific rotation of sodium scymnol sulphate is positive. Accordingly, it is concluded that the active principle isolated from shark is 24R-(+)-3α, 7α,12α,24,26-pentahydroxycoprostane-27-sodium sulphate ester.

The sodium or ammonium ion in the sulphate ester is easily replaced by other metal ions such as potassium, calcium, etc., or by organic amine cations

such as amino acids, etc., by means of well known procedures.

The following Tables illustrate the activity of the aqueous extracts of this invention:

TABLE I

	Dosage	Bioassay(A) (Units)	Bioassay(B) (Seconds)
Oil-soluble part of shark's liver	500mg	13,800	21
Water-soluble part of shark's liver	50mg	9,500	15
Control		13,000	22

TABLE II

Aqueous Extract of Shark's Gallbladder, Purified by:	Dosage	Bioassay(A) (Units)	Bioassay(B) (Seconds)
Charcoal adsorption	5mg		15
XAD-2 adsorption	1mg		16
Anion-exchange resin adsorption	0.5mg	8,200	14
Purified active principle	0.15mg	9,600	15
Control		14,000	22

Standard bioassays referred to in the above description were performed as follows:

Bioassay (A)

Biological test for protective activity against

carbon tetrachloride (CCl_4)-induced liver lesions in mice.

Male Std:ddy mice (weight 30-35g) were used in groups of 5 animals. Samples of test materials were administered orally 7 days at a suitable daily dose and 0.1ml of 5% CCl_4 in olive oil was orally administered at 24hrs after the last sample administration. Blood was obtained from the orbital sinus at 24hrs after the CCl_4 administration. Serum was obtained by centrifugation (3,000 rpm., 10min) and glutamic pyruvic transaminase (GPT) activity was measured by Reitman-Frankel-Momose method. Activity was expressed as a comparison of GPT values between the sample-administered groups and controls.

Bioassay (B)

Effect on respiration in nicotine administration to mice.

Male Std:ddy mice (weight 20-22g) were used in groups of 5 animals. Nicotine tartrate (3mg) was injected subcutaneously. Samples of test materials were orally administered 3hrs before nicotine administration. The time taken for 30 respirations was counted 5 minutes after nicotine administration. Activity was expressed as a comparison of the counted time between the sample-administered groups and controls.

The present invention also provides a pharmaceutical composition comprising an active substance as described above, together with a pharmaceutically acceptable carrier or diluent therefor. By way of example, the active substance can be formulated as stable tablets after being mixed as a

powder with a known carrier or bulking agent. Alternatively, the active substance can be incorporated into a lotion or cream base for topical application. In yet another alternative, the active substance can for example be filled in soft gelatin capsules, if desired after being admixed with shark's liver oil. Such pharmaceutical compositions may be used, for example, for the protection of the liver or activation of liver function in the treatment of diseases or conditions affecting the liver such as hepatitis, nephritis, diabetes, etc.. Such compositions may also be used for the activation of regeneration of skin tissue, for example, in the treatment of dermatitis, trauma or acne.

Clinical tests which have been performed using compositions containing the active substance have specifically demonstrated its activity in restoration of the liver function, and in the treatment of seborrhea.

In a further aspect of this invention, there is provided a dietary or health food composition which comprises the active principle described herein, together with one or more appropriate base or carrier materials. Such a composition may, for example, be useful in the treatment of a hangover.

In another aspect, the present invention provides a cosmetic composition comprising the active principle as described above, together with a cosmetic base material.

The compositions of the present invention may also incorporate known pharmaceuticals or other active ingredients, for example, antibiotics or other antibacterial substances.

Further details of this invention will be apparent from the following Examples which illustrate the invention without limiting it in any way.

EXAMPLE 1 - Preparation of Crude Active Principle

280g of a mixture of liver and gallbladder isolated from 4kg of shark was homogenised in 300ml of water, and the mixture was centrifuged at 12,000 rpm for 30 minutes to obtain a clear aqueous layer. 50g of ion exchange resin of basic anion exchange type was added to the aqueous layer and the mixture was left to stand overnight. The resin was removed by filtration and washed with water. The resin was then extracted with 200ml of 0.5% sodium chloride solution. 100g of XAD2 was added to the extracted solution. XAD2 was removed by filtration and washed with water. XAD2 was extracted with 200ml of ethanol. From the extract, ethanol was removed by distillation to obtain 45mg of crude active powder.

EXAMPLE 2 - Silica gel column chromatography

100g of crude active compound obtained by adsorption on a XAD-2 column was subjected to chromatography on a silica gel column, using MeOH-$CHCl_3$-H_2O(30:70:6) as solvent, to afford white powder (40g).

EXAMPLE 3 - Thin layer chromatography (TLC)

Crude active compound was subjected to TLC on a precoated silica gel 60 thin layer plate (Merck), using the system (parts by volume): n-BuOH(85)-AcOH(10)-H_2O(5) and MeOH(40)-$CHCl_3$(60)-H_2O(10). The active principle showed as a single spot

on TLC, and was visualized by spraying with vanillin sulfuric acid reagent.

EXAMPLE 4 - High performance liquid chromatography (HPLC)

Final purification of crude active powder was achieved by successive application of preparative HPLC with a reverse phase column. 31g of the active compound in the form of white powder, mp.140°, was obtained from 100g of XAD-2 purified sample. The approximate representative retention time of the active compound was 16 minute. The conditions for HPLC were as follows: column: YMC-Pack A-324(ODS); flow rate: 20ml/min.; mobile phase: CH_3CN-0.02N phosphate ammonium buffer (pH 7.45)(8:2); detector: refractive index.

EXAMPLE 5 - Column chromatography on Sephadex LH-20

Crude active compound (100g) obtained by adsorption on a XAD-2 column was subjected to gel filtration on Sephadex LH-20 column, using MeOH-$CHCl_3$(1:1) and then MeOH as eluents, to afford white powder (45g) from the MeOH fraction. Rechromatography on the same column afforded 30g of almost pure white powder.

EXAMPLE 6

Gall-bladders (65g), obtained from 5 sharks of the species Rhizoprionodon acutus (ca 8Kg weight), were homogenized and then freeze-dried. This material (10.25g) was used as a source of the active principle, sodium scymnol sulphate. After defatting the material with refluxing n-hexane (100ml x3), it was extracted with methanol (100ml x3) under reflux for 1h. The

concentrate (3.67g) was dissolved in H_2O (80ml), and applied to an Amberlite XAD-2 column (3.0 x 16.0cm). The column was eluted with H_2O (400ml) and then with ethanol (400ml). Then, the ethanol eluate (1.95g) was applied to Sephadex LH-20 column (3.0 x 32.0cm), chloroform and methanol (1:1). After elution with chloroform and methanol (200ml), the column was developed with methanol in batches of 50ml. Concentration of the methanol eluate containing the sodium salt gave a white gum (1.06g). Purification of this material (120mg) by HPLC yielded 85.6mg of sodium scymnol sulphate as white powder. The conditions for HPLC were as follows: column, YMC-Pack A-324(ODS) 10x300mm; flow rate, 2ml/min; mobile phase, 35% CH_3CN-0.1N Sodium Phosphate Buffer (pH 6.43); detector, Refractive Index, Sodium scymnol sulphate has the following physical data: White powder; $[\alpha]_D^{25}$ = 21.75(0.5c, in MeOH); Anal.: Calcd. for $C_{27}H_{47}O_9SNa$: C;56.82 H;8.30 S;5.62 Na;4.03. Found: C;56.99 H;8.79 S;5.62 Na;4.23. SIMS mass (m/e) : 654[$C_{27}H_{47}SO_9.HN(C_2H_6O)_2$], 574[$C_{27}H_{47}O_6.HN(C_2H_6O)_2$. IR$\nu_{max}^{KBr}$ cm^{-1} : 3420, 2950, 1470, 1380, 1230, 1070, 980, 910, 810. ^{1}H-NMR (in CD_3OD); δ(ppm): 4.22(1H,dd,J=4.5, 10.0Hz), 4.11(1H,dd,J=6.6, 10.0Hz), 4.00(1H, broad), 3.80(1H, m), 3.80-3.62(3H, m), 3.45-3.30(1H, m), 2.35-2.15(2H, m), 2.05-1.02(23H, m), 1.02(3H, d, J=6.2Hz), 0.92(3H, s), 0.72(3H, s). ^{13}C-NMR (in CD_3OD); δ(ppm): 74.1(d), 72.9(d), 71.4(d), 69.1(d), 66.7(t), 61.2(t), 48.3(d), 47.8(d), 47.5(s), 43.1(d), 43.0(d), 41.0(d), 40.3(t), 37.0(d), 36.5(t), 35.9(s), 35.8(t), 33.3(t), 32.1(t), 31.2(t), 29.5(t), 28.8(t), 27.9(d), 24.3(t), 23.2(q), 18.1(q), 13.1(q).

EXAMPLE 7

Trials have been conducted using the active principle of this invention in an antiseborrheous lotion applied topically by 40 male and female patients affected by long established (72) years facial hyperseborrhea. The trials were conducted as double blind trials with 20 patients applying a placebo and 20 patients applying the lotion containng the active principle.

In these trials, the treatment was applied three times daily (morning, midday and evening) over a period of 20 days, and an evaluation of seborrhea (Seborrhea Index) made at days 0, (prior to treatment), 10 and 21, (at end of treatment).

The results showed a significantly greater improvement in the seborrhea for patients using the lotion containing the active principle than for patients using the placebo. It was also observed that this improvement was shown in both male and female patients.

EXAMPLE 8 - Compositions

1. Cold cream

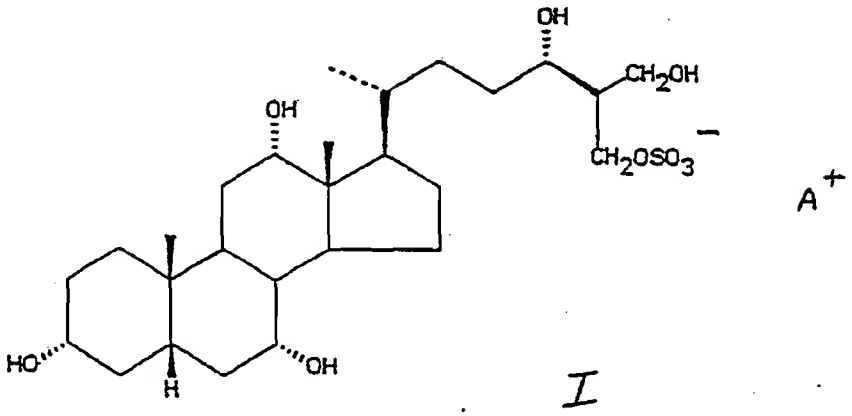
Spermacetti	6.0g
Beeswax	6.0g
Carbopol 934	10.0g
Sodium Carbonate	4.75g
Rose water	5.0ml
Rose oil	0.02ml
Expressed almond oil	56.0g
Active principle	0.05g
Distilled water	20.0g

2. Tonic

Ethanol	30ml
Active principle	20mg
Flavour	q.s.
Distilled water -	sufficient quantity to make 100ml

CLAIMS:

1. A compound of the general formula I, in substantially pure form,



I

wherein A is a cation.

2. A compound according to claim 1, wherein the cation is a sodium, potassium, calcium or ammonium ion, or an organic amine.

3. A method for the preparation of a compound of the general formula I as defined in claim 1, in substantially pure form, which comprises the steps of preparing an aqueous extract of the liver and/or gall-bladder of a shark, and isolating the said compound from said aqueous extract.

4. A method according to claim 3, wherein said step of isolation from the aqueous extract comprises at least one step selected from solvent extraction, adsorption and chromatography.

5. A pharmaceutical composition, comprising a compound of the general formula I as defined in claim 1, together with a pharmaceutically acceptable carrier or diluent therefor.

6. A pharmaceutical composition according to claim 5, in the form of a tablet, capsule, lotion or cream.

7. Use of a compound of the general formula I as defined in claim 1, for the protection of the liver or activation of liver function in the treatment of diseases or conditions affecting the liver.

8. A composition for the treatment of the skin comprising a compound of the general formula I as defined in claim 1, together with a topically acceptable carrier or diluent therefor.

9. A composition according to claim 8, further comprising an antibiotic or other antibacterial substance.

10. A cosmetic composition comprising a compound of the general formula I as defined in claim 1, together with a cosmetic base material.

11. Use of a compound of the general formula I as defined in claim 1, for the treatment of the skin.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00281

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07J 31/00, A61K 31/575

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
IPC	C07J 31/00
US Cl.	260/397.2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	US,A, 4296109 (LAURENT et al) 20 October 1981 (20.10.81)	(1)
A	US,A, 3994878 (PARTRIDGE, Jr. et al) 30 November 1976 (30.11.76)	(1)

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 November 1987 (17.11.87)	(30-11-87) 30 NOVEMBER 1987
International Searching Authority: Australian Patent Office	Signature of Authorized Officer: J.G. HANSON

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00281

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
US 4296109	AU 51423/79	CA 1127630	DE 2843690
	DK 4048/79	EP 10056	ES 484706
	GB 2034715	JP 55051100	SU 818489
	DE 2932166		
US 3994878	AT 7513/76	AT 5464/79	AT 5463/79
	AT 5465/79	BE 847131	CH 628907
	CH 634337	DE 2645527	FR 2351998
	FR 2407941	GB 1564806	GB 1564807
	GB 1564808	GB 1564809	GB 1564810
	IT 1068692	JP 52046061	NL 7611155
	CH 626096	CH 626097	US 4038272

END OF ANNEX

PA02-504

English abstract of reference A5

PATENT ABSTRACTS OF JAPAN

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A61K 7/00

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(71)Applicant : KANEBO LTD

(72)Inventor : SAKAI SHINGO
SAYO TETSUYA
YASUDA SUESHIGE
INOUE SHINTARO

(54) AGENT FOR INCREASING HYALURONIC ACID CONTENT OF HORNY LAYER

(57)Abstract:

PROBLEM TO BE SOLVED: To obtain the subject agent which can increase the hyaluronic acid content of the horny layer to optimize the water environment and hardness of the horny layer, allows to expect an effect for preventing rough skins and wrinkles, is stable in preparations, and is useful for skin cosmetics, by including N-acetylglucosamine.

SOLUTION: This agent for increasing the hyaluronic acid content of a horny layer comprises N-acetylglucosamine. The agent is preferably added to a skin cosmetic preferably in an amount of 0.001 to 10%, especially preferably 0.01 to 5%, based on the total amount of the cosmetic composition. The agent may preferably further be compounded with a polyhydric alcohol such as 1,3-butylene glycol, a vitamin derivative such as an ascorbic acid phosphoric acid ester salt, an anionic surfactant such as N-stearoyl-L-glutamic acid salt, and so on.

LEGAL STATUS

[Date of request for examination]

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[Date of final disposal for application]

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[Date of registration]

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[Date of extinction of right]

PAD2-504

English abstract of reference A6

PATENT ABSTRACTS OF JAPAN

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(43)Date of publication of application : 24.01.1984

(51)Int.Cl. A61K 7/00

(21)Application number : 57-122729

(22)Date of filing : 14.07.1982

(71)Applicant : SHISEIDO CO LTD

(72)Inventor : SHIMADA TADAHIRO

TOYODA HIDEKAZU

(54) COSMETIC

(57)Abstract:

PURPOSE: To provide a cosmetic containing amino sugars, N-acetylamino sugars or their salts existing in the crust of insects and crustaceans, and giving smoothness and moist feeling to the skin, and luster and finishing effect to the hair.

CONSTITUTION: The cosmetic contains 0.1W5.0wt% of one or more compounds selected from N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-glucosamine, D-galactosamine, their hydrochloride, sulfate, etc. The cosmetic has moistening effect, emmolient effect and skin activating effect, and gives smooth feeling, springiness and luster to the skin. It is also effective to moisten and soften the hair, and improves the combing property of the hair. The above compound can be obtained by decomposing the chitin of the crust of insects and crustaceans such as crab, mucopolysaccharide and sugar proteins, etc. of animals and vegetables, etc. with acid, alkali, enzyme, etc.

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[Date of request for examination]

[Date of sending the examiner's decision of rejection]

[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision of rejection]

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Original Article

Cholesterol sulfate and calcium affect stratum corneum lipid organization over a wide temperature range

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ABSTRACT

The main diffusion barrier for drugs penetrating through the skin is located in the intercellular lipid matrix in the upper layer of the skin, the stratum corneum (SC). The main lipid classes in the SC are ceramides (CER), free fatty acids (FFA) and cholesterol (CHOL). The lipids in SC are organized into two lamellar phases with periodicities of approximately 13 and 6 nm, respectively. Similar lipid organization has been found with equimolar CHOL:CER:FFA mixtures in SAXD studies performed at room temperature. However, one may conclude that the phase behavior of the mixtures is similar to that in SC only when the lipid organization of the lipid mixtures resembles that in SC over a wide temperature range. Therefore, in the present study, the organization of the lipid mixtures has been studied in a temperature range between 20° and 95°C. From these experiments it appeared that at elevated temperatures in equimolar CHOL:CER:FFA mixtures a new prominent 4.3 nm phase is formed between 35;–55°C, which is absent or only weakly formed in intact human and pig SC, respectively. As it has been suggested that gradients of pH and cholesterol sulfate exist in the SC and that Ca^{2+} is present only in the lowest SC layers, the effect of pH, cholesterol sulfate, and Ca^{2+} on the lipid phase behavior has been investigated with lipid mixtures. Both an increase in pH from 5 (pH at the skin surface) to 7.4 (pH at the SC;–stratum granulosum interface) and the presence of cholesterol sulfate promote the formation of the 13 nm lamellar phase. Furthermore, cholesterol sulfate reduces the amount of CHOL that is present in crystalline domains, causes a shift in the formation of the 4.3 nm phase to higher temperatures, and makes this phase less prominent at higher temperatures.

The finding that Ca^{2+} counteracts the effects of cholesterol sulfate indicates the importance of a proper balance of minor SC components for appropriate SC lipid organization. In addition, when the findings are extrapolated to the in vivo situation, it seems that cholesterol sulfate is required to dissolve cholesterol in the lamellar phases and to stabilize SC lipid organization. Therefore, a drop in cholesterol sulfate content in the superficial layers of the SC is expected to destabilize the lipid lamellar phases, which might facilitate the desquamation process.—**Bouwstra, J. A., G. S. Gooris, F. E. R. Dubbelaar, and M. Ponec.** Cholesterol sulfate and calcium affect stratum corneum lipid organization over a wide temperature range. *J. Lipid Res.* 1999. **40:** 2303;–2312.

Supplementary key words: stratum corneum, ceramides, phase behavior, X-ray diffraction

INTRODUCTION

One of the main functions of the skin is to act as a barrier for undesired exogenous substances. However, for topical application of drugs, this barrier forms the main problem. The skin barrier is located in the upper layer of the skin, the stratum corneum (SC) that is composed of keratin-rich cells surrounded by hydrophobic crystalline lipid lamellar domains. It has frequently been suggested that SC lipids play a dominant role in proper functioning of the skin barrier, as topically applied substances have often to pass the SC lipid regions that form a very dense structure, Therefore, a detailed knowledge about the SC lipid organization is of great importance. The main lipid classes present in the SC are ceramides (CER), cholesterol (CHOL), and free fatty acids (FFA). The ceramide fraction is composed of at least six ceramides, referred to as CER 1, CER 2 ... CER 6 (1). The FFA and CER both vary in hydrocarbon chain length, the main population of FFA and CER having an acyl chain length between 22 and 26 C atoms.

In diseased skin the barrier function is frequently impaired. There, a deviation in lipid composition has often been found. This includes *i*) a significant change in CER profile in atopic dermatitis and psoriatic scales (2) (3), *ii*) reduced FFA/CHOL and FFA/CER ratios in lamellar ichthyosis patients (4); and *iii*) 3- to 4-fold elevated levels of CSO_4 in recessive X-linked ichthyosis patients (5). It remains to be established whether the impaired barrier function can always be linked to a modulated lipid composition. In order to delineate the link between the barrier function and lipid composition, the role that individual lipid classes play in SC lipid phase behavior should be examined. Such studies with native tissue are hampered by the low availability of the required material from the diseased skin. In order to mimic diseased SC, one can consider to modulate lipid composition in SC isolated from normal skin. However this approach is not feasible, as it is impossible to selectively extract certain lipid classes from the SC. For this reason another approach, like the use of lipid mixtures isolated from SC, should be chosen.

Using X-ray techniques it has been established that in the SC the lipids are organized into two crystalline lamellar phases with periodicities of approximately 6 and 13 nm (6) (7) (8). A similar

organization has been observed using equimolar mixtures of CHOL, FFA, and CER isolated from pig SC, except that in these mixtures a larger proportion of CHOL appears in separate crystalline domains than observed in SC. These observations have been made in experiments performed at room temperature (9). In this study we have observed that in CHOL:CER mixtures, the phase behavior at room temperature is similar to that of intact SC. However, when the temperature-induced changes in phase behavior were examined, we observed that between 35° and 55°C a new phase with a periodicity varying between 4.3 and 4.5 nm was formed, which remained dominantly present with further increase of the temperature (9). In human SC this phase has not been encountered (8). The presence of such a phase at elevated temperatures indicates a lower stability of the lamellar phases in the lipid mixtures than in the SC. These observations illustrate the relevance for measuring temperature-induced phase changes, as differences in phase behavior at elevated temperature indicate that differences in lipid organization also exist at room temperature. Sometimes this is difficult to trace when performing lipid organization studies only at room temperature.

Not only the major SC lipids, CHOL, CER and FFA, but also minor fractions, like cholesterol sulfate (CSO_4) may affect SC lipid organization. The CSO_4 level throughout the SC does not remain constant, but reaches very low levels in the superficial SC layers (10) (11). In addition, a pH gradient exists in the SC (12) (13) (14) and Ca^{2+} also displays a characteristic gradient in the epidermis. Ca^{2+} level increases from the basal layers to the granulosum layer and declines again in the SC (15) (16) (17).

One of the methods to investigate the SC lipid organization is X-ray diffraction. However, X-ray diffraction is a bulk method that provides only information on overall lipid organization in the SC and does not allow study of the effects of local changes in pH and CSO_4 and Ca^{2+} contents on lipid organization in native SC. A systematic study can be performed with lipid mixtures prepared from isolated SC lipids. This allows experimental modulation in lipid composition to mimic the situation either at the stratum granulosum(SG);–SC interface (pH 7.4, high Ca^{2+} and high CSO_4 content) or in the superficial SC layers (pH 5;–6, low Ca^{2+} and low CSO_4 content). Because a drop in Ca^{2+} concentration already occurs in the lowest SC layers, but CSO_4 content decreases in the most superficial SC layer, the effect of CSO_4 in the absence of Ca^{2+} on the SC lipid organization is also of interest. In our present studies we chose to incorporate either 2% m/m or 10% m/m CSO_4 into equimolar CHOL:CER:FFA mixtures, to approximate the CSO_4 levels observed in normal and in recessive X-linked ichthyosis skin, respectively (5). Furthermore, as substantial amounts of Ca^{2+} are also present in superficial SC layers in psoriatic skin (18) and in skin covered for several weeks by a plaster after bone fracture (M. Ponec, unpublished results), we decided to study also the effect of Ca^{2+} at pH 5. Because at present no information is available on the local Ca^{2+} concentrations in the SC, we have chosen a concentration of 2 mmol Ca^{2+}, similar to that used to study the effect of Ca^{2+} on phospholipid membrane systems (19) (20) (21) (22) (23) being used as model systems for cell membranes.

MATERIALS AND METHODS

Isolation of stratum corneum from pig skin

Fresh pig skin was obtained from a slaughter house at the C.D.I. in Lelystad in the Netherlands and SC was isolated from the skin as described before (9).

Extraction, separation, and identification of lipids from stratum corneum

Epidermal lipids were extracted using the method of Bligh and Dyer (24). The extracted lipids were applied on a silicagel 60 (Merck) column with a diameter of 2 cm and a length of 33 cm. The various lipid classes were eluted sequentially using various solvent mixtures as published recently (9). The lipid composition of the collected fractions was established by one-dimensional high performance thin-layer chromatography, as described before (25). For quantification, authentic standards (Sigma) were run in parallel. The quantification was performed after charring using a photodensitometer with automatic peak integration (Desaga, Germany). Isolated CER fractions 1 to 6 were mixed to achieve the same ratio as found in the pig SC.

Preparation of lipid mixtures

The lipids were mixed in various molar ratios, using a mean CER molar weight of 700. For calculation of the mean ceramide molecular weight, the data on the ceramide composition and alkyl chain length distributions (26) were used. Mixtures were prepared from either *i*) CHOL, CER and FFA or *ii*) CHOL, CER, FFA and CSO_4 or *iii*) CHOL, CER, FFA and CSO_4 in the presence of 2 mM $CaCl_2$. For the FFA mixture, we chose long-chain free fatty acids in a molar ratio according to Wertz and Downing (1). The following fatty acids were included in the lipid mixtures: C16:0, C18:0, C22:0, C24:0, and C26:0 in a molar ratio of 1:3:42:37:7, respectively. The mixtures were prepared at either a pH of 7.4, which is the pH close to the stratum corneum;–stratum granulosum interface, or a pH 5, which is the pH of the skin surface (9) (10) (11). To achieve a pH of 7.4, a HEPES buffer (10 mmol, Na^+ based) was used. To prepare mixtures at a pH of 5, we used a citrate buffer (10 mmol, Na^+ based).

Small angle X-ray diffraction (SAXD)

All measurements were carried out at the Synchrotron Radiation Source at Daresbury Laboratory using station 8.2. The samples were put in a special designed sample holder with two mica windows. A detailed description of the equipment has been given elsewhere (7). The experimental conditions were similar as described before (9). The scattered intensities were measured as a function of θ, the scattering angle. Calibration of the detector was carried out with rattail and cholesterol. From the scattering angle the scattering vector (Q) was calculated $Q = 4\pi(\sin\theta)/\lambda$, in which λ is the wavelength being 0.154 nm at the sample position.

The diffraction pattern of a lamellar is characterized by a series of peaks at equal interpeak distance, $Q_n = n\,Q_1$, in which Q_n is the position of the nth order peak and Q_1 is the position of the 1st order peak. The periodicity can directly be calculated from the position of the peaks $d = n\,2\pi/Q_n$. The diffraction pattern of CHOL is characterized by two peaks at $Q = 1.87$ and $3.74\ nm^{-1}$.

The lipid phase behavior was also measured as a function of temperature. The temperature was

increased at a heating rate of 2°C/min between 25° and 95°C. Data collection was carried out continuously. Each minute a new diffraction curve was collected. In such a way each successive set of diffraction data reflects the mean phase behavior during a temperature rise of 2°C.

RESULTS

Phase behavior at room temperature

Mixtures prepared at pH 5. CHOL:CER:FFA mixture. The diffraction pattern of the equimolar CHOL:CER:FFA mixture is presented in (**Figure 1**) A. The spacings and corresponding periodicities are summarized in **Table 1**. As can be noticed from Table 1 and Figure 1a, this mixture formed two lamellar phases with periodicities of 13.0 and 5.4 nm, respectively. In addition, 3.36 and 1.69 nm diffraction peaks were observed that could be attributed to CHOL that separates as a phase in crystalline domains. The phase behavior of the equimolar CHOL:CER:FFA mixture was similar to that published recently (9).

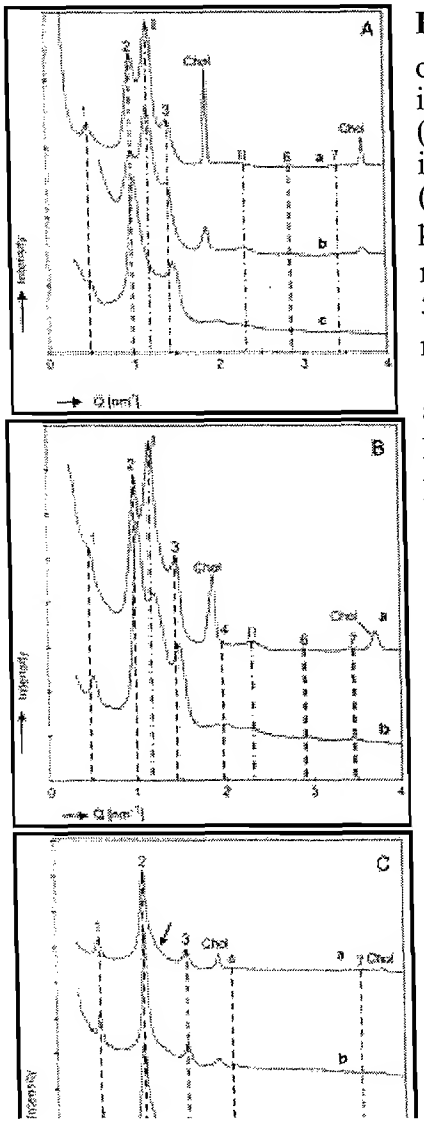

Figure 1. The effect of CSO_4 and Ca^{2+} on the phase behavior of equimolar CHOL:CER:FFA mixtures. The arabic numbers indicate the diffraction orders of the long periodicity phase (repeat distance between 12 and 13 nm). The roman numbers indicate the diffraction orders of the short periodicity phase (repeat distance between 5.3 and 5.5 nm). The diffraction patterns of the CHOL:CER:FFA:CSO_4 mixtures in molar ratios of A: (a) 1:1:1:0, (b)1:1:1:0.06 and (c) 1:1:1:0.3 at pH 5; B: (a) 1:1:1:0.06 and (b) 1:1:1:0.3 in the presence of 2 mmol Ca^{2+} at pH 5; C: (a) 1:1:1:0, (b) 1:1:1:0.06 and (c) 1:1:1:0.3 at pH 7.4. At curve (a) the shoulder indicated by an arrow refers to the first order diffraction peak of the short periodicity phase; D: (a) 1:1:1:0.06 and (b) 1:1:1:0.3 in the presence of 2 mmol in the presence of 2 mmol Ca^{2+} at pH 7.4.

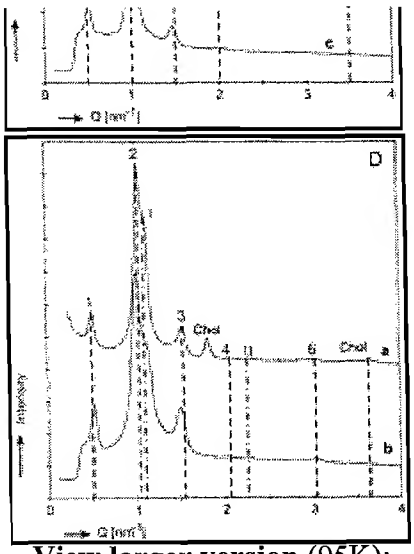

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Table 1. Effect of CSO_4 and Ca^{2+} ον τηΕ λιπιδ οΡganιzατιον ιν ∈θθιμολαΡ ΨΗΟΛΨΕΡΦΦΑ μιχτθΡΕσ ατ πΗ =

CHOL:CER:FFA:CSO_4 mixture. In order to study the effect of CSO_4 on the lipid phase behavior either 2% m/m or 10% m/m CSO_4 has been incorporated into the equimolar CHOL:CER:FFA mixture. Inspection of Table 1 and Figure 1a revealed that lipids in CHOL:CER:FFA:CSO_4 mixture in a molar ratio of 1:1:1:0.06 (2% m/m) formed two lamellar phases with periodicities of 12.8 and 5.4 nm, respectively, similar to that seen in the absence of CSO_4. Furthermore, the intensity of peaks attributed to crystalline CHOL decreased. A further increase in CSO_4 content to 10% m/m (molar ratio:1:1:1:0.3) induced a pronounced change in the lamellar phase behavior: the diffraction peaks attributed to the 5.4 nm phase and the peaks assigned to crystalline CHOL disappeared (Figure 1a).

CHOL:CER:FFA:CSO_4 and Ca^{2+}. The diffraction curve and corresponding spacings are depicted in Figure 1b and Table 1, respectively. Addition of 2 mmol $CaCl_2$ to a CHOL:CER:FFA:CSO_4 mixture in a molar ratio of 1:1:1:0.06 did not affect the peak intensities attributed to the 5.4 and 13.0 nm lamellar phases, but slightly increased the intensity of the CHOL reflections. This indicates that at low CSO_4 the presence of Ca^{2+} decreased the CHOL solubility in the lamellar phases. In contrast to that, addition of Ca^{2+} to 1:1:1:0.3 CHOL:CER:FFA:CSO_4 mixture did not result in the reappearance of the CHOL reflections. However, a reappearance of the 5.4 nm peak was observed.

The results at pH 5 demonstrate that CSO_4 increases the solubility of CHOL in the lamellar phases

and when present at high levels it promotes the formation of the long periodicity phase, while Ca^{2+} counterbalances partly the lipid phase changes induced by CSO_4.

Mixtures prepared at pH 7.4. CHOL:CER:FFA mixtures. The phase behavior of the equimolar CHOL:CER:FFA mixtures prepared at a pH of 7.4 is shown in Figure 1c and the corresponding spacings are presented in **Table 2**. From Figure 1c and Table 2 it is obvious that at pH 7.4 only one dominant lamellar phase was present, with a periodicity of 12.6 nm, which is in good agreement with our recent findings (27). The 6.2 nm peak (2nd order peak of the 12.5 nm phase) was asymmetric with a shoulder on its right-hand side, indicating the presence of an additional peak at a slightly shorter spacing (see arrow in Figure 1c). This shoulder is most likely the first order diffraction peak of the 5;–6 nm phase, which is prominently present at a pH 5 (see above). In addition, two diffraction peaks at 3.37 and 1.68 nm were observed that could be attributed to crystalline cholesterol.

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Table 2. Effect of CSO_4 and Ca^{2+} ον τηε λιπιδ οΡΓανιΖατιον ω ∈θθιμολαΡ ΨΗΟΛΨΕΡΦΦΑ μιχτυΡ∈σ ατ πΗ Ŧ>÷

CHOL:CER:FFA:CSO_4 mixture. Incorporation of 2% m/m CSO_4 into the equimolar CHOL:CER:FFA mixture caused a change of the 6.2 nm asymmetric peak into a symmetric one (2nd order peak of the 12.4 nm phase) and a slight reduction of the intensities of the CHOL reflections (Figure 1b). From these observations it is obvious that in the presence of 2% m/m CSO_4 the formation of the 5;–6 nm phase was suppressed and the solubility of CHOL in the lamellar phases was increased. An increase in CSO_4 content to 10% m/m increased the solubility of CHOL further. No further changes in the 12.5 nm lamellar phase were observed.

CHOL:CER:FFA:CSO_4 and Ca^{2+}. As shown in Table 2 and Figure 1d, addition of 2 mM Ca^{2+} to the 1:1:1:0.06 mixture of CHOL:CER:FFA:CSO_4 induced a reappearance of a 5.4 nm shoulder at the right-hand side of the 6.2 nm diffraction peak. Furthermore, in the presence of Ca^{2+}, the intensities of the peaks attributed to crystalline CHOL increased. Addition of Ca^{2+} to the 1:1:1:0.3 mixture of CHOL:CER:FFA:CSO_4 also caused a reappearance of the 5.4 nm shoulder, but no CHOL reflections were observed. The above results indicate that 2 mmol Ca^{2+} balances, at least partly, the changes in lipid phase behavior induced by CSO_4.

The results at pH 7.4 obviously show that CSO_4 increases the solubility of CHOL and promotes the formation of the long periodicity phase, while Ca^{2+} opposes the effect similar to that observed at pH 5.

Temperature-induced changes in the SC lipid phase behavior of mixtures prepared at pH 5
CHOL:CER:FFA mixtures. The phase behavior of the CHOL:CER:FFA mixtures has been followed as a function of temperature between 25° and 95°C (**Figure 2** A). Each curve represents the lipid

phase behavior during a 2°C rise in temperature. The phase behavior at room temperature is similar to that shown in Figure 1a. Two lamellar phases are present with periodicities 13.0 and 5.4 nm, respectively. The peaks attributed to the 5.4 and 13.0 nm phases start to decrease in intensity at approximately 55°C and disappear at about 63°C. At approximately 35;–39°C a new 4.3 nm peak is formed. A further temperature rise leads to a strong increase in the intensity of this peak and to a small shift in position to a spacing of 4.4 nm. Simultaneously with the increase in intensity of the 4.3 nm peak between 39° and 63°C, a 2.2 nm peak appears. This peak can most likely be attributed to the same phase, as it follows the positional and intensity changes of the 4.4 nm peak. At around 60°C the 4.4 nm peak transforms into a doublet that turns to a singlet again at 71°C. A further increase in temperature reduces the 4.4 nm and 2.2 nm peak intensity, but at 95°C the reflections are still present. The CHOL reflections start to decrease in intensity at approximately 37°C and disappear at around 55°C.

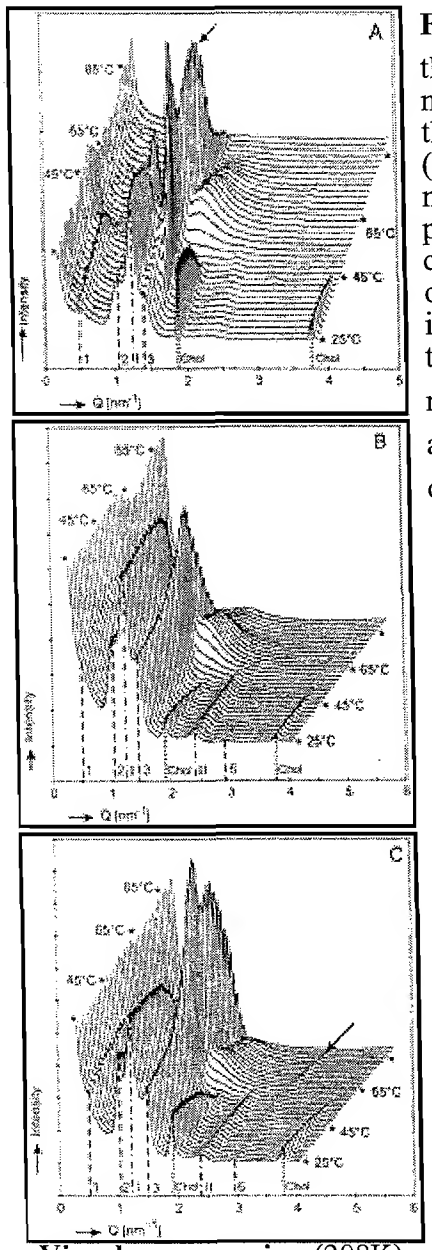

Figure 2. Temperature-dependent effect of CSO_4 and Ca^{2+} on the lipid phase behavior of CHOL:CER:FFA mixtures. The mixtures were prepared at pH 5. The arabic numbers indicate the diffraction orders of the long periodicity lamellar phase (repeat distance varies between 12.8 and 13 nm). The roman numbers refer to the diffraction peaks attributed to the short periodicity phase (repeat distance 5.4 nm). Each diffraction curve represents the scattered x-rays during a temperature rise of 2°C. A: Equimolar CHOL:CER:FFA mixture. The arrow indicates the formation of a new 4.3 nm phase at elevated temperatures. B: CHOL:CER:FFA:CSO_4 mixture in a molar ratio of 1:1:1:0.06. C: CHOL:CER:FFA:CSO_4 mixture with addition of 2 mmol $CaCl_2$. Note the arrow indicating the 2nd order reflection of the 4.4 nm phase.

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CHOL:CER:FFA:CSO_4 mixture. As depicted in Figure 2b, no changes in the lipid phase behavior have been noticed in the 1:1:1:0.06 CHOL:CER:FFA:CSO_4 mixture until the temperature reaches approximately 45°C. Between 50° and 55°C the CHOL diffraction peaks gradually reduced in intensity and subsequently disappeared at about 55°C. At about 60°C the intensity of the diffraction peaks attributed to the 5.4 and 12.8 nm lamellar phases started to decrease. In contrast to the observations made with the CHOL:CER:FFA mixture, no new 4.3 nm peak appeared at 35;–39°C, but the intensity of the 4.4 nm diffraction peak (3rd order of the 12.8 nm lamellar phase) started to increase at around 45°C. It is not clear whether in this temperature region it is caused by the formation of a new phase with a peak position at exactly the same spacing as the already existing peak or that the intensity increase is caused by a change in the lipid organization of the 12.8 nm phase. A further rise in temperature caused a further increase in the 4.4 nm peak intensity. A maximum peak intensity was reached at 61°C. The observation that the diffraction peaks attributed to the 5.4 and 12.8 nm phases disappear at 65°, while the 4.4 nm phase was still present, is indicative for the formation of a new 4.3 nm phase. The 4.4 nm peak disappeared at approximately 83°C.

As depicted in Figure 2c, the presence of 2 mmol Ca^{2+} had a profound effect on the phase behavior of the 1:1:1:0.06 CHOL:CER:FFA:CSO_4 mixtures at elevated temperature. Most substantial is the increase in 4.4 nm peak intensity that starts already at around 40°C. A further increase in temperature increased the peak spacing and peak intensity gradually. The peak intensity reached its maximum at approximately 67°C. Above this temperature the peak intensity and spacing decreased. At 95°C the peak (4.2 nm spacing) was still present. Because the 5.4 and 13 nm phases disappeared at 70°C, it is obvious that a new phase appeared at high temperature with a 2nd order peak at 2.15 nm (see arrow).

Our studies reveal that the presence of CSO_4 is required to mimic SC lipid phase behavior over a wide temperature range and that Ca^{2+} acts partly towards the CSO_4 effect not only at room temperature, but also at elevated temperatures.

DISCUSSION

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During epidermal differentiation, characteristic changes in lipid composition occur, consistent with the formation of a waterproof barrier. These changes include a progressive depletion of phospholipids and glucosphingolipids accompanied by enrichment in CER, CHOL, FFA, and small amounts of other polar (e.g., CSO_4) and nonpolar lipid species (cholesteryl esters and triglycerides). The relative amounts of CHOL, CER and FFA remain unchanged throughout the whole SC (M. Ponec and A. Weerheim, unpublished results). There is increasing evidence that within the SC also no significant change in

CER composition occurs (28).

The SC intercellular lipid bilayers originate from lamellar bodies that are abundantly present in the SG and are extruded to intercellular space at the SG/SC interface. After extrusion, the content of lamellar bodies is reorganized into lamellae. It has been suggested that the presence of Ca^{2+} is required for the formation of intercellular lamellae (29). The Ca^{2+} concentration is high at the SG/SC interface and in the first SC layers. Subsequently, it drops to very low levels in the upper SC. Not only the Ca^{2+} levels but also the pH and CSO_4 content decrease in the direction of the skin surface (11) (12) (13) (14) (30) (31).

The previous (27) and present studies have shown that the solubility of cholesterol in equimolar CHOL:CER:FFA mixtures increases in the presence of CSO_4 (**Figure 3**). Most likely the increased solubility of CHOL in the lamellar phases can be ascribed to the presence of the charged sulfate group. Due to the electric repulsion forces, the charged sulfate group increases the interfacial area per lipid molecule and thus reduces the lattice density and/or increases the chain mobility in the bilayers. This is in agreement with our recent findings (27) which showed that in the absence of CSO_4 the CHOL:CER:FFA mixtures form an orthorhombic phase, while upon addition of CSO_4 a liquid lateral packing appears. An increased fluidity has indeed been found in recessive X-linked ichthyosis by electron spin resonance (32). Studies of Kitson et al. (33) revealed that intercalation of CSO_4 in sphingomyelin bilayers increased the transition temperature of the lamellar to reversed hexagonal phase. They explained this phenomenon by an increase in the interfacial area. A stabilization of the membrane structure by CSO_4 has also been found in other phospholipid systems (21) (34).

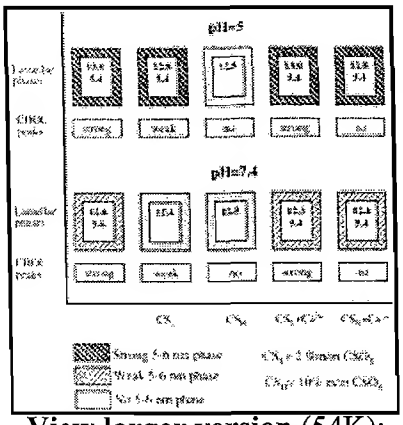

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Figure 3. A schematic presentation of the changes in the lipid phase behavior in equimolar CHOL:CER:FFA mixtures by changes of pH, Ca^{2+} (2 mmol), and CSO_4 content. The long periodicity phase, repeat distance of which varied between 12.4 and 13 nm, was always prominently present. The intensity of the 1st order diffraction peak attributed to the short periodicity phase (repeat distance varied between 5 and 6) varied strongly. This peak, indicative for the presence of the short periodicity phase, has been categorized in the following way: strong, weak and not present.

The formation of the long periodicity phase is also promoted by an increase in pH from 5 to 7.4. This pH change also increases the repulsion forces between the lipids and therefore is expected to reduce the lattice density and increase the chain mobility as well. These observations suggest a more general mechanism, namely that a reduction in lattice density or an increase in chain mobility promotes the

formation of the 12;–13 nm phase. This hypothesis is supported by at least two other recent observations. In SC isolated from reconstructed human epidermis and from murine skin, lattice density is reduced: in the former a hexagonal lateral packing is present (35), while in the latter a liquid phase coexists with an orthorhombic lateral packing (6). In both SC samples the 12;–13 nm lamellar phase dominates.

It has often been suggested that CSO_4 plays an important role in the desquamation process. Recently it has been shown that CSO_4 inhibits the enzymatic activity of enzymes responsible for desmosomal degradation (36). A drop in the CSO_4 content in the superficial SC layers (10) (11) will activate these enzymes and facilitate the desquamation process. However, not only the desmosomes, but also the lipid bilayers are responsible for the intercellular cohesion in the SC. The results of the present study demonstrate that a drop in the CSO_4 content will affect the SC lipid phase behavior. As observed from the measurements at elevated temperature (see below), the reduction in CSO_4 content decreases the stability of the bilayers and reduces the fraction of lipids arranged in a liquid lateral packing (27). The absence of this fluid phase is expected to reduce the elasticity of the lipid phases (37) and might even prevent the lamellae from following the contours of the corneocyte surfaces. This will decrease the cohesion between the cells and promote the desquamation process. Therefore, our data suggest that CSO_4 plays an important role in a proper functioning of the SC. In deeper SC layers CSO_4 increases the lipid mobility and, in the superficial layers, the decrease in CSO_4 levels promotes the desquamation process not only due to the increased desmosomal degradation (36), but also by destabilizing the lipid lamellar phases.

Changes induced by CSO_4 can be partly counterbalanced by the presence of 2 mmol Ca^{2+} (Figure 3). Namely, while 2% CSO_4 increases the solubility of CHOL in the lamellar phases, Ca^{2+} promotes formation of separate crystalline CHOL domains in the presence of 2% m/m CSO_4. Furthermore, while the 5–6 nm reflection at pH 7.4 disappears in the presence of 2 or 10% m/m CSO_4, in the presence of Ca^{2+} reappearance of the 5–6 nm reflection occurs. At pH 5 the same events are observed with 10% m/m CSO_4. These findings indicate that in the presence of 2 mmol Ca^{2+} the formation of the 5;–6 nm phase and the reformation of crystalline domains of CHOL is facilitated. However, in mixtures containing 10% m/m CSO_4, most probably the CSO_4 content is increased to such high levels that the formation of CHOL crystals is prevented. In phospholipid systems Ca^{2+} stimulates dehydration of phospholipid head groups and induces crystallization. This has been demonstrated for cardiolipin, phosphatidylserine, phosphatidylglycerol, and phosphatidylcholine (19) (20) (21) (22) (23). Because hardly any water is present between the bilayers in CER:CHOL:FFA mixtures it is unlikely that a similar mechanism is involved. Most likely Ca^{2+} is able to reduce the surface charge density induced by either CSO_4 or dissociated FFA (the pKa values of the fatty acids are approximately 6;–6.5 (38). This results in a reduction of the intermolecular electrostatic repulsion and an increase in lattice density. Whether Ca^{2+} links two opposing CSO_4 lipids as suggested in a previous paper (32) remains to be established. Consequently, when extrapolating these findings to the

situation in intact SC, changes induced by CSO_4 might be partly counterbalanced by Ca^{2+}. In normal skin this occurs only in the lowest SC layers where Ca^{2+} is present. In higher SC layers no Ca^{2+} could be detected (17). In contrast to this, CSO_4 levels remain unchanged up to the superficial SC cell layers. The observation that in about 50% of the human SC samples a small amount of CHOL phase separates (J. A. Bouwstra, G. S. Gooris, A. Weerheim, and M. Ponec, unpublished results) indicates that the level of CSO_4 is probably such that the SC lamellae are partially or completely saturated with CHOL. At the most superficial SC layer the CSO_4 level drops, which might induce a crystallization of CHOL and decrease the cohesion between the lipid lamellae.

At 10% m/m CSO_4 levels, only the 12;–13 nm phase is present. One can expect that in recessive x-linked ichthyosis skin, in which the CSO_4 level is increased from 3.4% w/w to 11.2% w/w (5), a change in the lipid phase behavior occurs. In a previous study (27) it has been shown that at low CSO_4 levels a fraction of lipids is arranged in a liquid lateral packing. One can speculate that a further increase in CSO_4 levels, as observed in recessive x-linked ichthyosis SC, might reduce the lattice density further and consequently might increase the permeability of the SC, which is in agreement with previous findings (32). The consequences of the presence of only the 12;–13 nm phase for the skin barrier function is yet unknown.

Phase behavior at elevated temperatures

Although it has been observed that at room temperature the CHOL:CER and CHOL:CER:FFA mixtures mimic the SC lipid organization quite closely, one may conclude that the phase behavior of the mixtures is similar to that in the SC only when the phase behavior of the lipid mixtures resembles that of SC over a wide temperature range. Furthermore, it has often been demonstrated that the lipid phase behavior is more sensitive to compositional changes at increased temperature than at room temperature (39). In a previous paper (9) we have reported that at increased temperature an additional phase, indicated by 4.3 nm spacing, is formed in CHOL:CER mixtures, which is more prominently present when the temperature is increased. This 4.3 nm phase has hardly ever been observed in SC derived from healthy persons and is only weakly present in pig SC (**Figure 4** A and B), but it has been observed in a pilot study in psoriatic scales (J. A. Bouwstra, unpublished results). From the experiments presented here it became clear that in the presence of only 2% m/m CSO_4 the appearance of the 4.3 nm phase shifts to higher temperatures and that the intensity of the corresponding diffraction peak reduces remarkably. This finding demonstrates that the SC lipid phase behavior is mimicked more closely in the presence of CSO_4 than in its absence. In addition, the 5.4 and 12.8 nm lamellar phases remain stable over a larger temperature range and the phases are less sensitive for temperature-induced changes. This clearly indicates that CSO_4 increases the stability of these phases. The situation is different when Ca^{2+} is also present. There the intensity of the 4.4 nm peak starts to increase at lower temperature, indicating that Ca^{2+} destabilizes the lamellar phases and counteracts the effect of CSO_4. Therefore an increase in Ca^{2+} level, such as present in psoriatic scales (18), might lead to destabilization of the SC lipid organization.

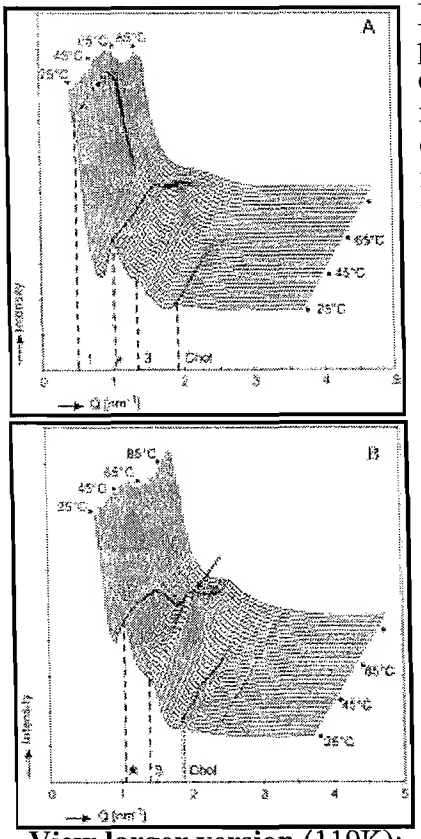

Figure 4. Temperature-induced changes in the diffraction pattern of pig and human SC. The arabic numbers indicate the diffraction orders of the 13 nm lamellar phase. The peak indicated by an asterisk is attributed to the 2nd order diffraction peak of the 13 nm phase and the 1st order of the 6 nm phase. Each diffraction curve represents the scattered X-rays during a temperature rise of 2°C. A: Human stratum corneum. Note the appearance of a new phase at around 50°C (see arrow). B: Pig stratum corneum. No new phase has been formed at elevated temperatures.

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To elucidate whether the stabilization of the lamellar phases by CSO_4 is caused by the introduction of the negative charge in the lamellae or whether it is due to the increased solubility of CHOL, we performed an additional experiment in which the CHOL content in CHOL:CER:FFA mixture (pH = 5) was reduced to such a level that no crystalline CHOL was present. In the 0.6:1:1 CHOL:CER:FFA mixture no separated crystalline domains of CHOL were detected, see **Figure 5** and increase in temperature up to 60°C did not affect the lipid phase behavior. At this temperature the diffraction peaks attributed to the 5.4 and 12.8 nm phases start to reduce in intensity and finally disappear between 60° and 70°C. In the same temperature region, a very broad diffraction peak is observed that disappears at approximately 75°C. No increase in the intensity of the 4.4 nm peak has been observed in this mixture. When extrapolating these results to 1:1:1:0.06 CHOL:CER:FFA:CSO_4 mixture, it became clear that in the diffraction pattern of this mixture the reduction in the 4.4 nm peak intensity at elevated temperatures is not caused by the presence of an additional negative charge introduced by CSO_4, but can be ascribed to the decrease in crystalline CHOL that separates as a phase at room temperature. This obviously demonstrates that the increased stability of the lamellar phases induced by CSO_4 is based on its capacity to enhance the solubility of CHOL in the lamellar phases.

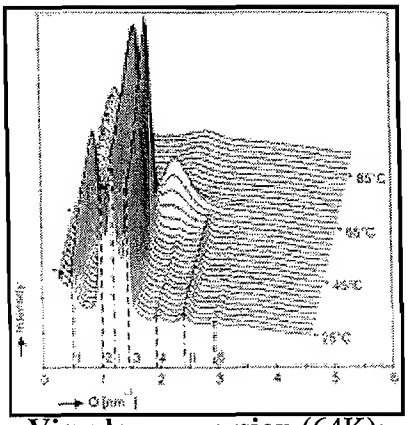

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Figure 5. Temperature-induced changes in diffraction patterns of the CHOL:CER:FFA mixture prepared in a molar ratio of 0.6:1:1 at pH 5. Each curve represents the phase behavior during a temperature rise of 2°C. The arabic numbers indicate the diffraction orders of the 12.8 nm. The roman numbers indicate the diffraction orders of the 5.4 nm lamellar phase.

In conclusion, despite a low CSO_4 level present in SC of normal skin, CSO_4 might be important in stabilizing the lamellar phases in the deeper layers of the SC. Furthermore, its absence in the superficial layers of SC might destabilize the lipid lamellae and therefore facilitate the desquamation process.

Manuscript received May 24, 1999; and in revised form August 31, 1999 **Abbreviations:** CHOL, cholesterol; CER, ceramides; FFA, free fatty acids; CSO_4, cholesterol sulfate; λ, wave length; θ, scattering angle; d, periodicity; SC, stratum corneum

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Interaction between corneocytes and stratum corneum lipid liposomes in vitro

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Small unilamellar vesicles were made from a mixture of epidermal ceramides (45%), cholesterol (35%), free fatty acids (15%) and cholesteryl sulfate (5%). Isolated corneocytes prepared from pig epidermis were added to the liposomes and the interaction between corneocytes and liposomes was studied by (1) thin-section electron microscopy and (2) monitoring the release of aqueous contents of the vesicles by following the fluorescence intensity of carboxyfluorescein entrapped in the vesicles. The vesicles adsorbed readily onto the corneocytes and slowly transformed into lamellar sheets. Enhanced fluorescence intensity indicated a corneocyte-induced membrane fusion process that resulted in the release of aqueous contents of the vesicles. The results suggest a cohesive role for the corneocyte cell envelope, which consists of a monomolecular layer of lipids covalently bound to the outside of a cross-linked protein envelope. This may be one of the major factors in the reassembly of extruded membranous disks into lamellar sheets which occurs during the final stages of epidermal differentiation.

Introduction

The stratum corneum (SC) of the mammalian epidermis consists of keratinized cells embedded in an extracellular matrix of multiple lipid lamellae [1]. These intercellular membranous sheets of the SC constitute the epidermal water barrier [2] and are known to originate from the lamellar bodies found in the cells of the granular layer of the epidermis [3,4]. The lamellar bodies contain stacks of membranous disks which appear to be flattened vesicles [4]. After their discharge into the intercellular space, the disks are reassembled to form the intercellular lamellar sheets of the SC. During this process, the lipids undergo drastic biochemical changes as the cells progress from the granular layer to the horny layer, and unlike other biological membranes, the SC lipid lamellae have been shown to be virtually devoid of phospholipids [5]. Recently, we have demonstrated the ability of relatively non-polar lipid mixtures (ceramides, cholesterol, free fatty acids and cholesteryl sulfate) of the SC to form liposomes in vitro [6] and investigated some of the factors that cause the fusion of these vesicles into lamellar sheets [7–9].

Interactions of liposomes with a range of biological membranes and cell types have been widely studied in cell biology to provide models for membrane-mediated biophysical processes such as fusion and cell-cell adhesion [10–13]. The recent discovery in our laboratory of a chemically bound lipid envelope surrounding corneocytes [14] suggested a cohesive interaction between the lipid envelope and the intercellular lipid lamellae. In the present study we investigated whether the interaction between corneocytes and SC lipid liposomes may have a role in promoting transformation of the extruded lamellar disks into the intercellular lamellae of the stratum corneum.

Materials and Methods

Lipids. Epidermal ceramides were isolated by preparative thin-layer chromatography from total lipid extracts of pig epidermis as described previously [15]. A mixture of free fatty acids was prepared by combining carnauba wax fatty acids [16] with palmitic acid to a ratio of 8 : 2 by weight, forming a mixture close to the distribution of free fatty acids in stratum corneum [17]. Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO). Cholesteryl sulfate was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and purified chromatographically.

Abbreviations: CF, carboxyfluorescein; SC, stratum corneum; SUV, small unilamellar vesicle; TMA, trimethylamine.

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Corneocytes. Corneocytes were prepared from pig SC as described previously [18]. Pieces of epidermis were obtained from freshly killed pigs by heating the skin to 60–65°C for 1 min. The epidermis was then peeled off and digested in 0.5% trypsin (Sigma Chemical Co., St. Louis, MO, Type III) in phosphate-buffered saline (PBS) at pH 7.5 at 4°C overnight. After rinsing in distilled water, the tissue pieces were treated with fresh trypsin/PBS solution for 2 h at room temperature. Pieces of SC thus obtained were washed in distilled water and treated with a detergent solution containing 8 mM *N,N*-dimethyldodecylamine oxide (Procter and Gamble, Cincinnati, OH) and 2 mM sodium dodecyl sulfate (Mallinckrodt, Paris, KY) in PBS at 45°C for 24 h. SC sheets disintegrated into a mixture of individual corneocytes and empty corneocyte envelopes that were separated by centrifugation in cesium chloride solution of density 1.28 [18]. The corneocytes were washed in ethanol to remove the detergents, resuspended in 1 mM sodium azide and stored at 4°C until further use.

Preparation of liposomes. Appropriate volumes of solutions of individual lipids in chloroform/methanol (2:1, by vol.) were combined to obtain a mixture containing 45% by weight of ceramides, 35% cholesterol, 15% free fatty acids and 5% cholesteryl sulfate. Liposomes were prepared from these lipid mixtures in an aqueous buffer containing 100 mM NaCl, 5 mM Tris, 1 mM NaN_3 and 1 mM EDTA by sonication, as described elsewhere [6]. The total amount of lipid in the suspension was 5 mg/ml. The final pH of the dispersions was adjusted to 7.5 by dialysis. In the fusion assay experiments liposomes were prepared in a buffer containing 100 mM carboxyfluorescein (CF), 5 mM trimethylamine (TMA) and 0.5 mM EDTA. Vesicles were separated from nonencapsulated CF by gel filtration on Sephadex G-50. The elution buffer contained 5 mM TMA and 0.5 mM EDTA and was at pH 9, same as that of the liposome suspension. The final pH of the eluted suspensions were adjusted to pH 7.5 by dialysis.

Interaction of liposomes with corneocytes. Corneocytes were added to the liposomes as a suspension in Tris buffer at 37°C to a final concentration of 10 mg/ml. Dry weight of the corneocytes was determined after drying an aliquot of the aqueous cell suspension. The corneocytes were kept in suspension by bubbling nitrogen at the rate of 0.1 ml per min. All the suspensions were maintained at 37°C until analysis by electron microscopy. For fusion assay experiments, corneocytes were added as a suspension in TMA buffer and incubated at 37°C for 1 h in the cell used for fluorescence measurement and the corneocytes were kept in suspension by stirring. The final concentration of the suspension was 1.5 mg of lipid and 1 mg of corneocytes per ml.

Electron microscopy. Control liposomes were analyzed by freeze-fracture electron microscopy as described previously [6]. The corneocyte-containing dispersions were analyzed by thin-section electron microscopy 1 h, 24 h and 1 week after the addition of corneocytes. The dispersions were centrifuged in a microfuge at 10000 × *g* and the wet pellets were fixed in 0.2% RuO_4 in cacodylate buffer for 15 min at room temperature. The fixed pellets were preembedded in agar, dehydrated in graded acetones, embedded in Spurr's resin and sectioned. Silver-gold sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H-7000 electron microscope operating at 75 kV.

Fusion Assay. The release of vesicle contents to the external medium induced by the corneocytes was measured by monitoring fluorescence intensity of carboxyfluorescein (CF), encapsulated initially at a high self-quenching concentration in the vesicles. When the entrapped CF is released to the outside medium, CF is diluted and the fluorescence is directly proportional to the concentration of CF [19,20]. Fluorescence was measured in an Aminco-Bowman fluorometer using excitation and emission wavelengths of 492 nm and 580 nm, respectively. Fluorescence was measured before and after the disruption of the vesicles by the addition of Triton X-100 (0.2% v/v). CF-containing vesicles were incubated at 37°C with corneocytes for 1 h and fluorescence was measured before and after the addition of Triton. The vesicles were also incubated at 37°C with the supernatant buffer from the corneocyte suspension for 1 h and fluorescence was measured before and after the addition of Triton. Relative fluorescence intensities were corrected for dilution after the addition of cell suspensions and TX-100. CF concentrations were determined from calibration curve generated from fluorescence intensity measurements of CF solutions prepared in TMA buffer at pH 7.5.

Results

Fig. 1 shows a freeze-fracture micrograph of control liposomes at pH 7.5. These liposomes were small unilamellar and ranged in size from 20 to 200 nm. The liposomes remained stable for several weeks. Figs. 2A and 2B show the thin-section electron micrographs (TEMs) of corneocytes prepared from pig epidermis. Fig. 2A shows isolated corneocytes as well as some in contact with each other. Fig. 2B shows two corneocytes in contact with each other. The cell envelope consists of a protein envelope seen as a broad electron-dense band; outside of this is an electron-lucent band corresponding to the covalently linked lipids [14]. Corneocytes in contact are seen to be separated by two electron-lucent bands, representing their respective lipid envelopes in contact with each other. Figs. 3A–3D show the TEMs of corneocytes that were added to the liposome dispersion. Fig. 3A shows small unilamellar vesicles (SUV) adsorbed on to the corneocyte lipid envelope, which

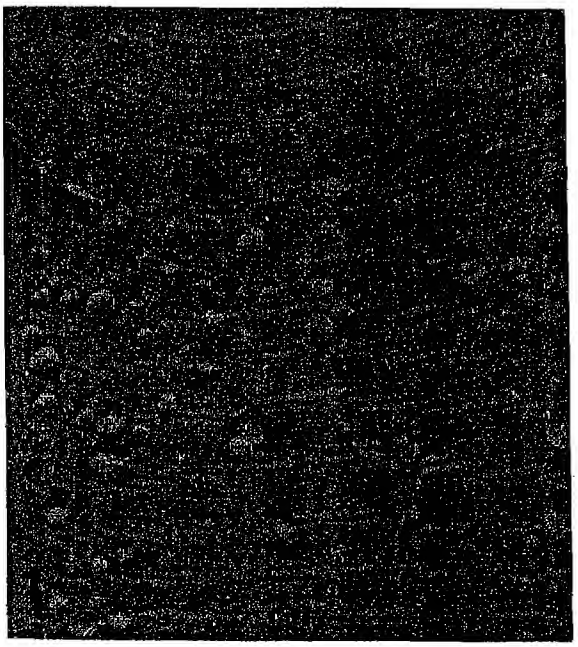


Fig. 1. Freeze-fracture electron micrograph of control liposomes. Arrowhead indicates the direction of shadowing. Bar = 200 nm.

3B shows regions of multiple lamellae similar to those seen in the intercellular region of natural SC. Fig. 3C shows two corneocytes with their intercellular region filled with multiple lamellae formed from the added liposomes. There were regions where the SUVs were adsorbed onto fragments of cell envelope, as seen in Fig. 3D. There were some isolated SUVs that remained stable and unadsorbed even after a week of incubation with corneocytes at 37 °C (not shown).

Release of carboxyfluorescein from SC lipid vesicles under various conditions is shown in Table I. Fluorescence obtained after lysis of the vesicles with Triton X-100 was taken as the value for 100% release. Initial fluorescence of the vesicles was taken as 0% release, which was 50 to 60% of the maximal value, indicating a high initial release of CF from these vesicles. The high initial release of the aqueous contents is due to the history of the vesicles. It was found necessary to use a high pH (≥ 9) during sonication to facilitate the dispersion of these relatively nonpolar lipids in the presence of large quantity (100 mM) of CF. The vesicles were then dialyzed to pH 7.5 after the removal of nonencapsulated CF. Amount of CF encapsulated at pH 9 and 7.5 were found to be 3.77 and 1.28 nmol/μmol of

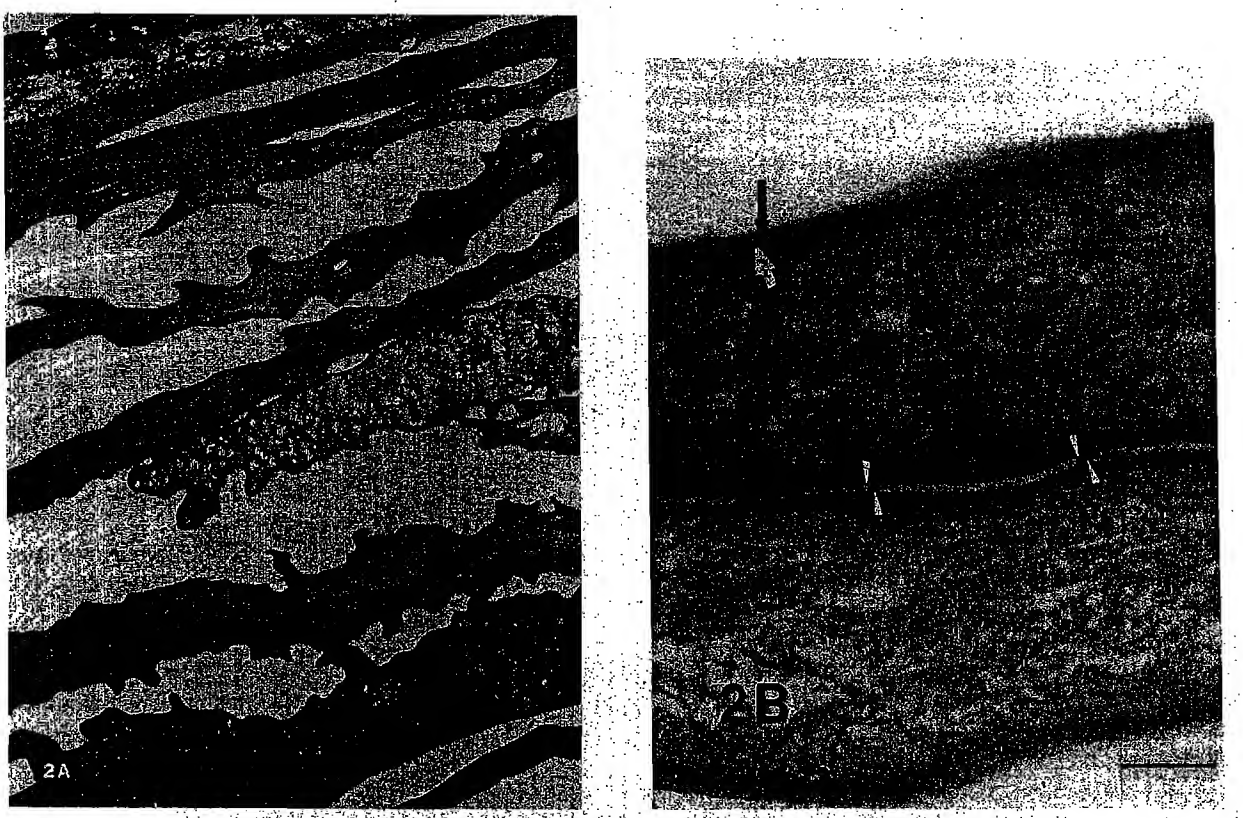
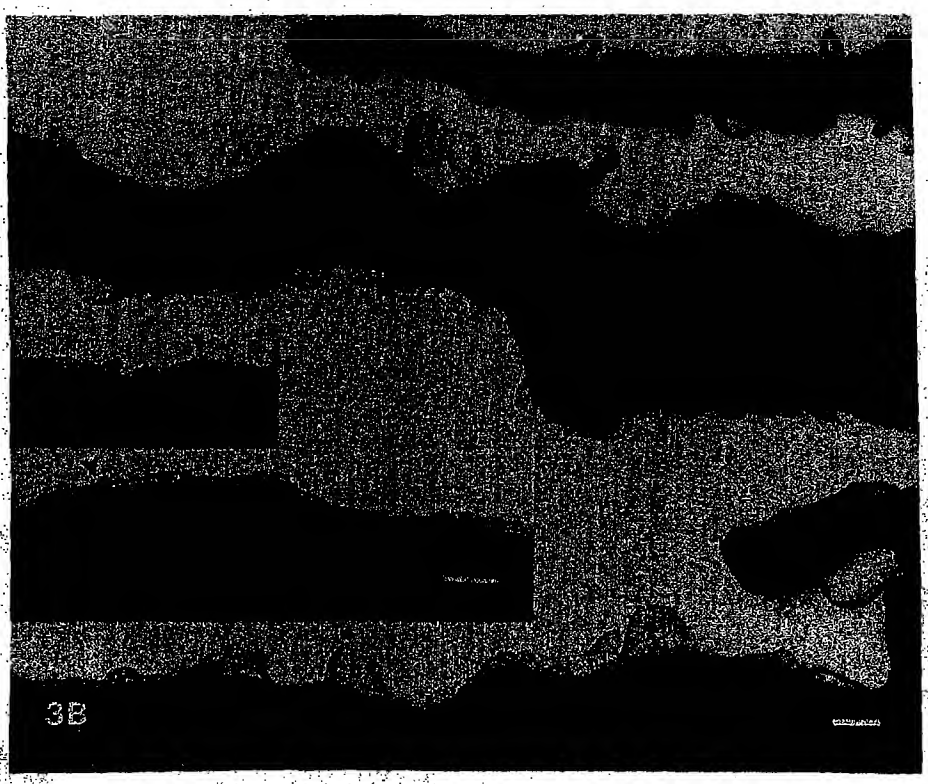


Fig. 2. Thin-section electron micrographs of control corneocytes. The corneocytes were fixed in ruthenium tetroxide and embedded in Spurr's resin. The sections were stained in uranyl acetate and lead citrate. (A) Bar = 200 nm. (B) White arrow shows the protein envelope. Black arrow shows the lipid envelope. Arrowheads show the lipid envelopes from adjacent corneocytes in contact with each other. Bar = 100 nm.


3A


3B

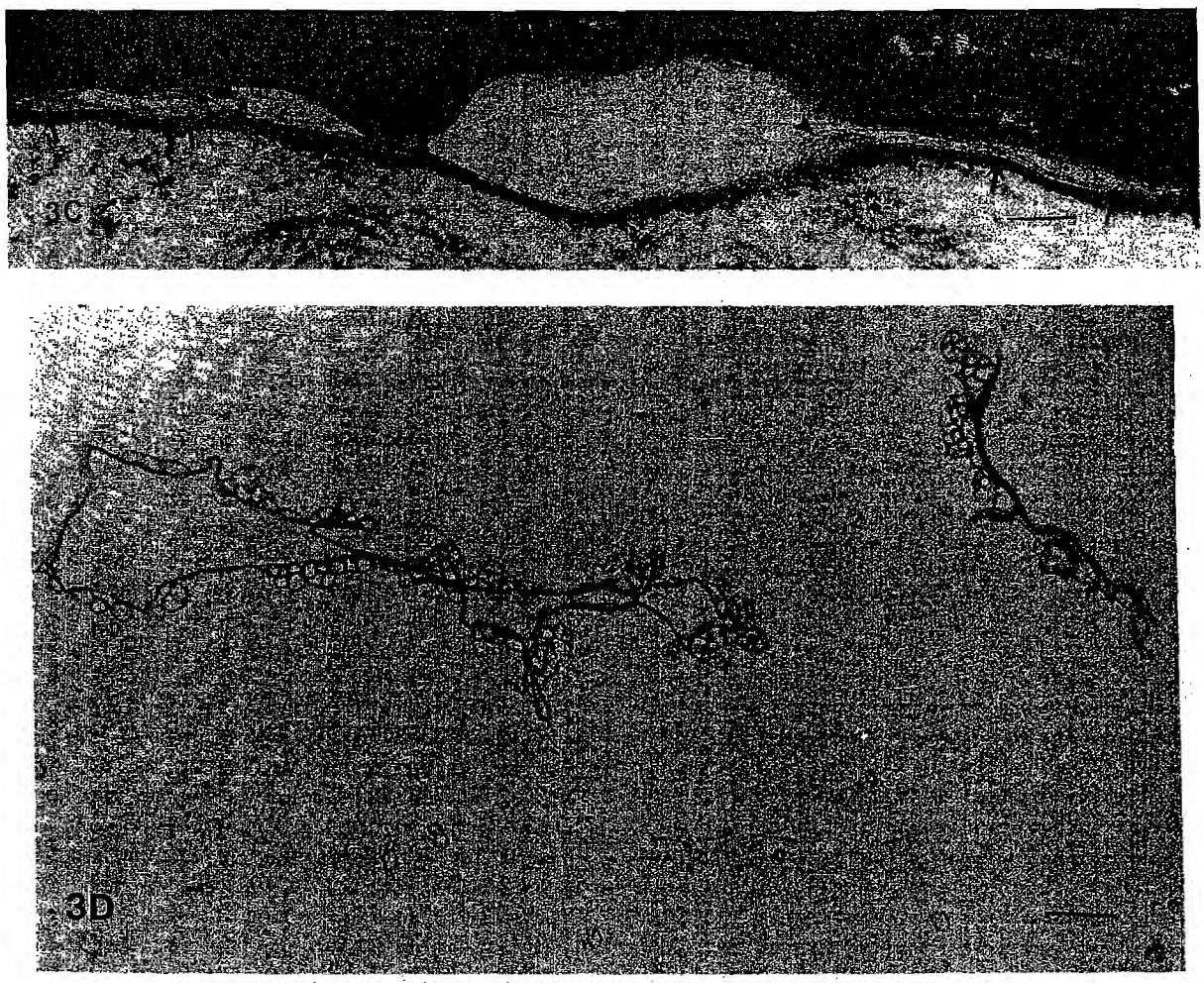


Fig. 3. Thin-section electron micrographs of corneocytes added to the liposomes. Incubation time was 1 h. (A) The liposomes are seen adsorbed onto the corneocytes. Bar = 200 nm. (B) Liposomes are seen at different stages of transformation. Bar = 100 nm. Inset shows multiple lamellae formed from the adsorbed liposomes (arrowheads). Bar = 50 nm. (C) Arrows show the intercellular region filled with multiple lamellae formed from the adsorbed liposomes. Arrowhead shows remnants of a partially formed lamella from the adsorbed liposome. Bar = 100 nm. (D) Liposomes are seen adsorbed onto the exterior of empty cell envelopes. Bar = 500 nm.

lipid, respectively, as shown in Table I. Incubation of the vesicles with supernatant buffer from the corneocytes suspension resulted in some release of entrapped CF (10–12%) while incubation with corneocytes under similar conditions resulted in enhanced release of CF (35–40%) as shown in Table I.

Discussion

Liposomes formed from SC lipids were extremely stable at pH 7.5. The high curvature of these SUVs, the repulsive hydration forces, and the electrostatic forces of the charged headgroups (of the partially ionized fatty acids and the completely ionized cholesteryl sulfate) apparently are sufficient to prevent them from undergoing any fusion or aggregation.

The corneocyte cell envelope has been thought of as a cross-linked protein envelope beneath the horny cell 'plasma membrane' [21,22]. These are usually seen as in Fig. 2B, respectively, as a broad electron-dense band and a narrow electron-lucent band in TEMs. Recently, the electron-lucent band has been shown to consist of a monomolecular layer of lipids, predominantly made up of ω-hydroxyacylsphingosines, that are covalently bound to the protein envelope [14]. These corneocyte lipid envelopes have been proposed to have a cohesive role in regions of cell-cell contact, especially in SC sheets that have been depleted of the intercellular lamellae by extensive extraction with chloroform/methanol [14], as well as in SC that was heated above the 75 °C transition temperature of the SC lipids (unpublished observations). The corneocyte lipid envelopes from contiguous

TABLE I

Release of carboxyfluorescein from SC liposomes at 37°C

	CF released (nmol/μmol of lipid)	
	during incubation with cells/supernatant [a]	after lysis with Triton [b]
Vesicles at pH 7.5		1.28±0.19
Vesicles at pH 9.0		3.77 [c]
Vesicles incubated for 1 h with corneocytes at pH 7.5	0.44±0.06	1.23±0.18
Vesicles incubated for 1 h with supernatant (buffer) at pH 7.5	0.16±0.03	1.45±0.18

[a] Calculated as the difference between the amount of CF released from the vesicles after 1 h of incubation with cells/supernatant and the amount of CF released before the addition of cells/supernatant (initial release).

[b] Calculated as the difference between the amount of CF released after the addition of Triton and the amount of CF released before the addition of cells/supernatant and Triton (initial release).

[c] Mean of two measurements. $n = 4$ for rest of the data.

corneocytes are seen as two lucent bands in regions where isolated corneocytes have come in contact with each other (Fig. 2B), supporting a cohesive role for the corneocyte lipid envelope.

When corneocytes were added to the liposomes, the vesicles adsorbed onto the surface of the corneocytes as seen in Fig. 3A. The liposomes remained adsorbed even after the washing and rinsing procedures during sample preparation for electron microscopy, indicating the stability of the adsorption. Stable adsorption of phospholipid liposomes to different cells has been demonstrated by a variety of techniques [10]. Such adsorption may be mediated either by biochemical forces (surface receptors, antibodies, etc.), by purely physical forces (electrostatic, hydration, hydrophobic, etc.) or by a combination of both types of forces.

SC has until recently been thought to be devoid of surface receptors such as lectins [23,24]. Recently, a 40 kD a glycoprotein has been isolated and has been localized to the corneocyte lipid envelope [25]. Brysk et al. have suggested that this endogenous lectin could play a major role in the cell cohesion in SC [26]. However, the same authors have shown that the membrane glycoproteins are solubilized by the nonionic detergent used in the corneocyte preparation [26]. Thus, in corneocytes prepared by detergent treatment of SC, it is reasonable to assume that it is purely a physical force that causes the cell-cell cohesion, as well as the adsorption of SC lipid liposomes on the surface of the corneocytes.

The amount of CF released after the incubation of vesicles with corneocytes is about three times that caused by the incubation of the vesicles with the buffer alone, suggesting a cell-induced process which results in an enhanced release of the aqueous contents. Adsorption of vesicles onto the corneocytes would not render the vesicles leaky. Enhanced release of CF from vesicles treated with corneocytes suggests a fusion process that renders the vesicles more leaky, presumably resulting from the collapse of the vesicles during or after the fusion process. CF release data along with the appearance of lamellae on corneocytes treated with SC lipid liposomes, as seen in Figs. 3B and 3C, suggests the formation of these lamellae from the adsorbed liposomes by a membrane fusion process. While some of the adsorbed liposomes transform to lamellae within the first hour of incubation, this transformation appears to be an inefficient process judging by the large number of liposomes that remained adsorbed on the corneocytes even a week after the addition of corneocytes to the liposomes. This is not very surprising in the absence of fusogens that are known to promote aggregation and fusion of SC lipid liposomes [7–9], which include calcium [7], epidermal acylceramides and acylglucosylceramides [8,9], low pH (< 6.5) and drying [27]. Presence of large number of adsorbed liposomes and corneocyte surfaces covered with lamellae and the independence of the relative amounts of corneocyte surface with adsorbed liposomes versus cell surface with lamellae suggests an alternative mechanism of formation of lamellar structures by direct transformation of unadsorbed vesicles occurring concomitant to and independent of adsorption process. We do not have any experimental evidence to distinguish between the two mechanisms of formation of the lamellae.

The adsorption of SC lipid liposomes onto corneocytes and their transformation into lamellar sheets in the absence of any of these known fusogens indicates an independent role for the corneocyte lipid envelope in these processes. Two plausible mechanisms can be postulated for the transformation of SUVs into lamellar sheets on the basis of the appearance of these systems in TEM. After initial adsorption of the liposomes, there could be a transfer of lipids from the liposomes to the corneocyte lipid envelope. Such a transfer can be facilitated through transient intermediary structures like mixed micelles that can be formed between partially ionized fatty acids and other lipid components or through a direct transfer of lipids at the regions of adsorption. This would lead to incorporation of parts of the vesicle bilayer into the lipid envelope. This may also be accompanied by fusion of the adsorbed liposomes with each other while they are immobilized by adsorption and are in close proximity with each other. This would lead to the collapse of one layer of adsorbed liposomes and their transformation into a bilayer segment to the cell envelope, seen as two electron dense bands, as in Fig. 3B.

In regions where multilamellar structures are attached to the corneocyte lipid envelope (Figs. 2B

3C), the fusion mechanism may involve flattening of the adsorbed and/or unadsorbed liposomes and their subsequent edge-to-edge fusion to form multilamellar structures. Corneocytes, which are flat and elongated, with the molecularly smooth lipid envelope on their surface would act as a template for the initial flattening and for the subsequent transformation of flattened vesicles into extended lamellar structures. It should be informative to see if SC lipid liposomes are adsorbed and/or lamellar structures are formed on delipidized corneocytes. However, the alkaline hydrolysis procedure used for the removal of the ester-linked ω-hydroxyceramides resulted in completely delipidized corneocytes in which the protein envelopes were partially separated from the cell contents [14]. These delipidized corneocytes disintegrated easily and could not be used for any further study.

The observations made in the present study suggest a cohesive role for the corneocyte lipid envelope in the transformation of SC lipid liposomes into lamellar sheets. This role may be one of the major factors in reassembly of the extruded membranous disks into lamellar sheets which occurs in the intercellular space between the upper granular layer and the horny layer during the final stages of epidermal differentiation [3,28].

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